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(57) Abstract

Vaccines and immunogenic compositions which contain at least one immunogenic antigenic determinant, the antigenic determinant being fused to a Hepatitis B virus core antigen and heterologous thereto and methods for making same are provided.

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COMPOSITIONS OF ANTIGEN CONTAINING RECOMBINANT SALMONELLA, THEIR USE IN ANTI-MALARIAL VACCINES AND METHOD FOR THEIR PREPARATION

Cross-Reference to Related Applications

This application is a continuation-in-part of copending U.S. Application No. 07/868,950 filed April 13, 1992 which is a continuation-in-part of U.S. application No. 07/785,748 filed November 7, 1991, which is a continuation-in-part of U.S. Application No. 07/612,001, filed November 9, 1990; which is a continuation-in-part of U.S. Application Serial No. 200,934, filed June 1, 1988, which is a continuation-in-part of copending U.S. Application Serial No. 058,360, filed June 4, 1987; U.S. Application Serial No. 200,934 is also a continuation-in-part of copending U.S. Application Serial No. 251,304, filed October 3, 1988, which is a continuation-in-part of copending U.S. Application Serial No. 106,072, filed October 7, 1987. This application is also a continuation-in-part of U.S. Serial No. 331,979, filed March 31, 1989.

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These applications are hereby incorporated herein by reference.

Field of the Invention

This invention relates to avirulent microbes expressing recombinant protozoan antigens, their method of preparation, and their use in vaccines. More specifically, it relates to avirulent Salmonella that express immunogenic antigens of Plasmodium.

Background of the Invention

Malaria continues to be a widespread and debilitating human disease that is caused by a protozoan parasite, Plasmodium spp., injected by mosquitoes of the genus Anopheles. The most commonly fatal species of Plasmodium in humans is P. falciparum. Various forms of treatment or prevention of malaria are known, but, heretofore, an effective vaccine preventing the disease has not been developed.

Studies involving genetic and protein analysis of Plasmodia have determined that certain repeat sequences in the circumsporozoite (CS) proteins of Plasmodia are immunodominant antibody recognition sites in plasmodial infection. It has also been shown that antibodies raised against CS proteins confer protection against experimental P. falciparum challenge. The use of CS proteins directly as a vaccine is limited because of absence of a T-cell epitope and also because purified, native CS is difficult and expensive to produce and the recognition of CS, at least in mice, is MHC restricted. It has also proved to be difficult to express the entire CS protein in a prokaryotic host. Such a vaccine would also require parenteral administration, and thus is not amenable for mass vaccination purposes, particularly in underdeveloped nations that have a relatively high incidence of malarial infection.

In the design of an effective vaccine, it is desirable to provide optimal delivery to the immune system to produce a maximal antibody response. This consideration has resulted in the development of recombinant hybrid fusion proteins which have incorporated viral epitopes into highly immunogenic proteins such as hepatitis B core (HBcAg) (Clarke et al., Nature, London 330, 381-384, 1987) or surface (HBsAG) (Delpeyroux et al., Science, 223, 472-475, 1986) antigen particles, the Ty element of yeast (Adams et al., Nature, London, 329, 68-70, 1988) or poliovirus virions (Burke et al., Nature, London, 332, 81-82, 1988). Each of these studies discuss the incorporation of viral epitopes into an immunogenic structure for use as a viral vaccine (Clarke 1987), but do not address the viability or usefulness of such an approach to combat a protozoan-based disease, such as malaria.

It would, therefore, be advantageous to provide an effective anti-malarial vaccine which is capable of providing protective immunity.

Brief Description of the Invention

Oral vaccines utilizing live avirulent derivative of a pathogenic microorganisms have several advantages. For example, they are economically desirable in that they eliminate the cost of purification of the immunogenic antigens. Also, they involve non-invasive techniques for administration, and thus are more suitable to mass vaccination programs. Another advantage is that an oral vaccine delivers replicating organisms to the mucosal immune system where local responses are maximally stimulated.

Attenuated Salmonella, such as S. typhi, S. typhimurium, or S. choleraesuis are attractive candidates to serve as carrier vaccines for the expression of Plasmodium antigens and for their delivery to the human immune system. The resulting vaccines may be bivalent, and confer

protection against Salmonella-based disease and Plasmodium infection, as well as to other enteric bacteria with which antibodies to Salmonella cross react. However, a critical prerequisite for successfully using this approach in immunizing humans is that there must exist highly immunogenic yet safe attenuated strains of Salmonella to deliver the plasmodial antigens to the immune system. In addition, the plasmodial antigens should be stably expressed in the avirulent derivative of a pathogenic strain, and be capable of eliciting protective immune responses in the immunized individual.

Accordingly, one embodiment of the invention is a composition comprised of live avirulent Salmonella that expresses at least one recombinant immunogenic epitope of Plasmodium.

Another embodiment of the invention is an immunogenic composition comprised of live avirulent Salmonella that expresses at least one recombinant immunogenic epitope of Plasmodium wherein the immunogenic epitope is one from the circumsporozoite proteins of Plasmodium, and wherein the Salmonella also expresses a region encoding HBV core antigen (HbcAg) to yield a polypeptide that forms a particle, and wherein the Salmonella is a Δcya Δcrp Δcdt mutant.

In another embodiment of the invention, the Salmonella in the immunogenic compositions of the above embodiments are also Δasd mutants, and the polypeptides encoding the plasmodial epitopes are expressed from a vector also encoding aspartate semialdehyde dehydrogenase (Asd), such that loss of Asd expression also causes loss of expression of the polypeptides comprised of the Plasmodium epitopes.

Yet another embodiment of the invention is a method of preparing a vaccine comprising providing a composition comprised of live avirulent Salmonella that expresses at

least one recombinant immunogenic epitope of Plasmodium, and mixing the composition with a suitable excipient.

Brief Description of the Drawings

Figure 1 is an illustration of the oligonucleotide sequences which encode the amino acid sequences of the CS repeat sequences of P. falciparum and P. berghei.

Figure 2 is an illustration of the structure of the HBc-CS repeat hybrids prepared in accordance with the teachings of this invention.

Figure 3 is a graph which shows the recovery of CFU from the Peyer's patches of 8 week old BALB/c mice at specified times after peroral inoculation with 9 x 10^8 CFU of χ3622 (Δ[crp-cyaG] -10), 1 x 10^9 CFU of χ3737 (pSD110'/Δ[crp-cyaG] -10) and 1 x 10^9 CFU of χ3339 (wild type). Three mice were sacrificed for each time point. The results are given as geometric means ± standard deviations.

Figure 4 is a graph which shows the recovery of CFU from the spleens of 8-week-old BALB/c female mice at specified times after peroral inoculation with 9 x 10^8 CFU of χ3622 (Δ[crp-cyaG] -10 1 x 10^9 CFU of χ3737 (pSD110'/Δ[crp-cyaG] -10) and 1 x 10^9 CFU of χ3339 (wild type). Three mice were sacrificed for each time point. The results are given as geometric means ± standard deviations.

Figure 5 is a partial restriction map of pYA1077. The 1.0 kb M. leprae insert DNA fragment from λgt11 clone L14 was subcloned into the EcoRI site of pYA292. There is a single asymmetrical SalI site within the M. leprae insert DNA. There are no sites within the M. leprae insert DNA for the following restriction endonucleases: BamHI, HindIII, PstI, and XbaI.

Figure 6 is a half-tone reproduction showing a Western blot of proteins produced by S. typhi, S. typhimurium, and E. coli strains harboring pYA1077 and

pYA1078. The proteins on the nitrocellulose filters were reacted with pooled sera from 21 lepromatous leprosy patients. Lane 1, molecular size markers (sizes are indicated to the left of the blot); Lane 2, proteins specified by S. typhi χ4297 with pYA292; Lanes 3 to 5, proteins specified by three independent S. typhi χ4297 isolates each containing pYA1078; Lanes 6 to 8, proteins specified by three independent isolates of S. typhi χ4297 isolates each containing pYA1077; Lane 9, proteins specified by S. typhimurium χ4074 with pYA1077; Lane 10, proteins specified by E. coli χ6060 with pYA1075 (a pUC8-2 derivative containing the 1.0 kb M. leprae DNA insert from λgt11 clone L14 in the same orientation relative to the lacZ promoter as it is in pYA1077). Note: the immunologically reactive protein specified by pYA1075 is slightly larger than that specified by PYA1077 because it is a fusion protein with the alpha region β-galactosidase.

Figure 7 is a half-tone reproduction showing a Western blot of proteins produced by λgt11::M. leprae clone L14 and S. typhi, S. typhimurium and E. coli strains harboring pYA292, pYA1077 and pYA1078.

Figure 8 is a graph showing the growth of wild-type and mutant strains of S. typhi Ty2 and ISP1820 at 37°C in human sera.

Figure 9 is a half-tone reproduction showing a Western blot of proteins produced by S. typhimurium expressing HBc-CS genes.

Figure 10 is a plasmid map of pYBC75CS1.

Figure 11 is a plasmid map of pYBC75CS2.

Modes for Carrying Out the Invention

Invasive yet attenuated Salmonella are desirable carrier microorganisms for the delivery of antigens to the mucosal and systemic immune systems by the oral route. In the current invention, avirulent derivative of a pathogenic (also referred to as attenuated) strains of Salmonella are used as carrier organisms for the expression of immunogenic

Plasmodium antigens from recombinant DNA constructs. The *Salmonella* expressing the immunogenic recombinant antigens are useful for, *inter alia*, the preparation of multi-valent oral vaccines.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., Maniatis, Fritsch & Sambrook, MOLECULAR CLONING; A LABORATORY MANUAL, Second Edition (1989); DNA CLONING, VOLUMES I AND II (D.N. Glover ed. 1985); OLIGONUCLEOTIDE SYNTHESIS (M.J. Gait ed, 1984); NUCLEIC ACID HYBRIDIZATION (B.D. Hames & S.J. Higgins eds. 1984); TRANSCRIPTION AND TRANSLATION (B.D. Hames & S.J. Higgins eds. 1984); ANIMAL CELL CULTURE (R.I. Freshney ed. 1986); IMMOBILIZED CELLS AND ENZYMES (IRL Press, 1986); B. Perbal, A PRACTICAL GUIDE TO MOLECULAR CLONING (1984); the series, METHODS IN ENZYMOLOGY (Academic Press, Inc.); GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (J.H. Miller and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory), Methods in Enzymology Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, Eds., respectively), Mayer and Walker, eds. (1987), IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY (Academic Press, London), Scopes, (1987), PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, Second Edition (Springer-Verlag, N.Y.), and HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, VOLUMES I-IV (D.M. Weir and C.C. Blackwell eds. 1986). All patents, patent applications, and publications mentioned herein, both supra and infra, are hereby incorporated herein by reference.

As used herein, a polynucleotide "derived from" a designated sequence refers to a polynucleotide sequence which is comprised of a sequence of approximately at least about 6 nucleotides, preferably at least about 8 nucleotides, more preferably at least about 10-12

nucleotides, and even more preferably at least about 15-20 nucleotides corresponding to a region of the designated nucleotide sequence. "Corresponding" means homologous to or complementary to the designated sequence. Regions from which typical polynucleotide sequences may be "derived" include but are not limited to, for example, regions encoding specific epitopes, as well as non-transcribed and/or non-translated regions.

The derived polynucleotide is not necessarily physically derived from the nucleotide sequence shown, but may be generated in any manner, including for example, chemical synthesis or DNA replication or reverse transcription or transcription. In addition, combinations of regions corresponding to that of the designated sequence may be modified in ways known in the art to be consistent with an intended use.

Similarly, a polypeptide or amino acid sequence "derived from" a designated nucleic acid sequence refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 3-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence. This terminology also includes a polypeptide expressed from a designated nucleic acid sequence. The term "polypeptide" refers to the primary amino acid sequence of a protein; polypeptides may be subsequently modified by modifications known within the art, for example, phosphorylation, glycosylation, intra-disulfide bonding, and still be within the definition of "polypeptide".

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence. It may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant

expression system, or isolation from a microorganism. A recombinant or derived polypeptide may include one or more analogs of amino acids or unnatural amino acids in its sequence. Methods of inserting analogs of amino acids into a sequence are known in the art. It also may include one or more labels, which are known to those of skill in the art.

The term "recombinant polynucleotide" as used herein intends a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of a polynucleotide with which it is associated in nature, (2) is linked to a polynucleotide other than that to which it is linked in nature, (3) does not occur in nature, or (4) is not in the form of a library.

The term "polynucleotide" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA and RNA. It also includes known types of modifications, for example, labels which are known in the art, methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example proteins (including for e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide.

The term "purified polynucleotide" refers to a polynucleotide which is essentially free, i.e., contains less than about 50%, preferably less than about 70%, and even more preferably less than about 90% of polypeptides with which the polynucleotide is naturally associated. Techniques for purifying polynucleotides from bacteria are known in the art, and include for example, disruption of the bacteria with a chaotropic agent, differential extraction and separation of the polynucleotide(s) and polypeptides by ion-exchange chromatography, affinity chromatography, and sedimentation according to density.

"Recombinant host cells", "host cells", "cells", "cell lines", "cell cultures", and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be, or have been, used as recipients for recombinant vectors or other transfer DNA, and include the progeny of the original cell which has been transfected. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

A "replicon" is any genetic element, e.g., a plasmid, a chromosome, a virus, a cosmid, etc. that behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control.

A "vector" is a replicon in which another polynucleotide segment is attached, so as to bring about the replication and/or expression of the attached segment.

"Control sequence" refers to polynucleotide sequences which are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoters, ribosomal binding sites, and terminators; in eukaryotes, generally, such control

sequences include promoters, terminators and, in some instances, enhancers. The term "control sequences" is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example, leader sequences.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

The term "expression vector" as used herein refers to a vector in which a coding sequence of interest is operably linked to control sequences.

A "recombinant gene", as used herein, is defined as an identifiable segment of polynucleotide within a larger polynucleotide molecule that is not found in association with the larger molecule in nature. The recombinant gene may be of genomic, cDNA, semisynthetic, or synthetic origin.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a bacterial gene, the gene will usually be flanked by DNA that does not flank the bacterial gene in the genome of the source bacteria. Another example of the heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

As used herein, "DAP" refers to both stereoisomers of diaminopimelic acid and its salts, i.e., both the LL- and meso- forms, unless otherwise shown by specific notation.

The gene symbols for mutant strains utilized herein are those described by Bachmann (1987), and Sanderson and Roth (1987). The symbols used for transposons, particularly Tn*10*, follow the convention described in Bukhari et al. (1977).

An "individual" treated with a vaccine of the invention is defined herein as including all vertebrates, for example, mammals, including domestic animals and humans, various species of birds, including domestic birds, particularly those of agricultural importance. In addition, mollusks and certain other invertebrates have a primitive immune system, and are included as an "individual".

"Transformation", as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, for example, direct uptake, electroporation, transduction, or conjugation. The exogenous polynucleotide, may be maintained as a non-integrated vector; such as a plasmid, or alternatively, the total or part of the polynucleotide may be integrated within the host genome.

As used herein, a "*phoP* gene or its equivalent" refers to a gene which encodes a product which regulates the expression of other genes, including loci encoding virulence attributes (for example, facilitating colonization, invasiveness, damage to an infected individual, and survival within macrophages or cells in the immune defense network), and including a gene encoding a phosphatase, for e.g., *phoN* in *Salmonella*.

Organisms which may contain a "*phoP* gene or its equivalent" include all members of the family Enterobacteriaceae (e.g., *E. coli*, *Salmonella*, *Proteus*,

Klebsiella, Serratia, Providencia, Citrobacter, Edwardsiella, Hafnia, and Enterobacter), members of other bacterial genera (e.g., Staphylococcus, Rhizobium, Mycobacterium, Aerobacter, Alcaligenes, and Bacillus, and several Candida species. The phoP product is a regulator of acid phosphatases [Kier et al. (1979)].

As used herein, a "pathogenic microorganism" causes symptoms of a disease associated with the pathogen.

An "avirulent microorganism" also referred to as an avirulent derivatilve of a pathogenic microorganism is one which has the ability to colonize and replicate in an infected individual, but which does not cause disease symptoms associated with virulent strains of the same species of microorganism. Avirulent does not mean that a microbe of that genus or species cannot ever function as a pathogen, but that the particular microbe being used is avirulent with respect to the particular animal being treated. The microbe may belong to a genus or even a species that is normally pathogenic but must belong to a strain that is avirulent. Avirulent strains are incapable of inducing a full suite of symptoms of the disease that is normally associated with its virulent pathogenic counterpart. Avirulent strains of microorganisms may be derived from virulent strains by mutation.

The term "microbe" as used herein includes bacteria, protozoa, and unicellular fungi.

A "carrier" microbe is an avirulent microbe as defined above which contains and expresses a recombinant gene encoding a protein of interest. As used herein, a "carrier microbe" is a form of a recombinant host cell.

An "antigen" refers to a molecule containing one or more epitopes that will stimulate a host's immune system to make a secretory, humoral and/or cellular antigen-specific response. The term is also used interchangeably with "immunogen."

A "hapten" is a molecule containing one or more epitopes that does not itself stimulate a host's immune system to make a secretory, humoral or cellular response.

The term "epitope" refers to a site on an antigen or hapten to which an antibody specific to that site binds. An epitope could comprise 3 amino acids in a spatial conformation which is unique to the epitope; generally, an epitope consists of at least 5 such amino acids, and more usually, consists of at least 8-10 such amino acids. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site."

An "immunological response" to a composition or vaccine is the development in the host of cellular and/or antibody-mediated immune response to the composition or vaccine of interest. Usually, such a response consists of the subject producing antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells directed specifically to an antigen or antigens included in the composition or vaccine of interest.

A "vertebrate" is any member of the subphylum Vertebrata, a primary division of the phylum Chordata that includes the fishes, amphibians, reptiles, birds, and mammals, all of which are characterized by a segmented bony or cartilaginous spinal column. All vertebrates have a functional immune system and respond to antigens by producing antibodies.

The term "protein" is used herein to designate a naturally occurring polypeptide. The term "polypeptide' is used in its broadest sense, i.e., any polymer of amino acids (dipeptide or greater) linked through peptide bonds. Thus, the term "polypeptide" includes proteins, oligopeptides, protein fragments, analogs, muteins, fusion proteins and the like.

An "open reading frame" (ORF) is a region of a polynucleotide sequence which encodes a polypeptide; this

region may represent a portion of a coding sequence or a total coding sequence.

A "coding sequence" is a polynucleotide sequence which is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to mRNA, cDNA, and recombinant polynucleotide sequences.

"Immunologically identifiable with/as" refers to the presence of epitope(s) and polypeptide(s) which are also present in the designated polypeptide(s). Immunological identity may be determined by antibody binding and/or competition in binding; these techniques are known to those of average skill in the art, and are also illustrated infra.

A polypeptide is "immunoreactive" when it is "immunologically reactive" with an antibody, i.e., when it binds to an antibody due to antibody recognition of a specific epitope contained within the polypeptide. Immunological reactivity may be determined by antibody binding, more particularly by the kinetics of antibody binding, and/or by competition in binding using as competitor(s) a known polypeptide(s) containing an epitope against which the antibody is directed. The techniques for determining whether a polypeptide is immunologically reactive with an antibody are known in the art. An "immunoreactive" polypeptide may also be "immunogenic". As used herein, the term "immunogenic polypeptide" is a polypeptide that elicits a cellular and/or humoral immune response, whether alone or linked to a carrier in the presence or absence of an adjuvant.

As used herein, the term "antibody" refers to a polypeptide or group of polypeptides which are comprised of

at least one antibody combining site. An "antibody combining site" or "binding domain" is formed from the folding of variable domains of an antibody molecule(s) to form three-dimensional binding spaces with an internal surface shape and charge distribution complementary to the features of an epitope of an antigen, which allows an immunological reaction with the antigen. An antibody combining site may be formed from a heavy and/or a light chain domain (HV and VL, respectively), which form hypervariable loops which contribute to antigen binding. The term "antibody" includes, for example, vertebrate antibodies, hybrid antibodies, chimeric antibodies, altered antibodies, univalent antibodies, the Fab proteins, and single domain antibodies.

"Treatment" as used herein refers to prophylaxis and/or therapy.

By "immunogenic" is meant an agent used to stimulate the immune system of a living organism, so that one or more functions of the immune system are increased and directed towards the immunogenic agent. Immunogenic agents include vaccines. Immunogenic agents can be used in the production of antibodies, both isolated polyclonal antibodies and monoclonal antibodies, using techniques known in the art.

By "vaccine composition" is meant an agent used to stimulate the immune system of a living organism so that protection against future harm is provided. "Immunization" refers to the process of inducing a continuing high level of antibody and/or cellular immune response in which T-lymphocytes can either kill the pathogen and/or activate other cells (e.g., phagocytes) to do so in an organism, which is directed against a pathogen or antigen to which the organism has been previously exposed. Although the phrase "immune system" can encompass responses of unicellular organisms to the presence of foreign bodies, e.g., interferon production, in this application the phrase is restricted to the anatomical features and mechanisms by

which a multi-cellular organism produces antibodies against an antigenic material which invades the cells of the organism or the extra-cellular fluid of the organism. The antibody so produced may belong to any of the immunological classes, such as immunoglobulins A, D, E, G or M. Immune response to antigens is well studied and widely reported. A survey of immunology is given in Barrett, James T., Textbook of Immunology: Fourth Edition, C.V. Mosby Co., St. Louis, MO (1983).

As used herein, the "sense strand" of a nucleic acid contains the sequence that has sequence homology to that of mRNA. The "anti-sense strand" contains a sequence which is complementary to that of the "sense strand".

As used herein, the term "probe" refers to a polynucleotide which forms a hybrid structure with a sequence in a target region, due to complementarity of at least one sequence in the probe with a sequence in the target region. The polynucleotide regions of probes may be composed of DNA, and/or RNA, and/or synthetic nucleotide analogs.

As used herein, the term "target region" refers to a region of the nucleic acid which is to be amplified and/or detected. The term "target sequence" refers to a sequence with which a probe or primer will form a stable hybrid under desired conditions.

The term "primer" as used herein refers to an oligomer which is capable of acting as a point of initiation of synthesis of a polynucleotide strand when placed under appropriate conditions. The primer will be completely or substantially complementary to a region of the polynucleotide strand to be copied. Thus, under conditions conducive to hybridization, the primer will anneal to the complementary region of the analyte strand. Upon addition of suitable reactants, (e.g., a polymerase, nucleotide triphosphates, and the like), the primer is extended by the polymerizing agent to form a copy of the

analyte strand. The primer may be single-stranded, or alternatively may be partially or fully double-stranded.

The terms "analyte polynucleotide" and "analyte strand" refer to a single- or double-stranded nucleic acid molecule which is suspected of containing a target sequence, and which may be present in a biological sample.

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from an individual, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs, and also samples of in vitro cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, putatively virally infected cells, recombinant cells, and cell components).

As used herein, the term "oligomer" refers to primers and to probes. The term oligomer does not connote the size of the molecule. However, typically oligomers are no greater than 1000 nucleotides, more typically are no greater than 500 nucleotides, even more typically are no greater than 250 nucleotides; they may be no greater than 100 nucleotides, and may be no greater than 75 nucleotides, and also may be no greater than 50 nucleotides in length.

The term "coupled" as used herein refers to attachment by covalent bonds or by strong non-covalent interactions (e.g., hydrophobic interactions, hydrogen bonds, etc.). Covalent bonds may be, for example, ester, ether, phosphoester, amide, peptide, imide, carbon-sulfur bonds, carbon-phosphorus bonds, and the like.

The term "support" refers to any solid or semi-solid surface to which a desired polypeptide or polynucleotide may be anchored. Suitable supports include glass, plastic, metal, polymer gels, and the like, and may take the form of beads, wells, dipsticks, membranes, and the like.

The term "label" as used herein refers to any atom or moiety which can be used to provide a detectable (preferably quantifiable) signal, and which can be attached to a polynucleotide or polypeptide.

In the invention, avirulent microbes containing recombinant construct(s) of DNA encoding antigen(s) comprised of one or more immunogenic epitopes of Plasmodium are used for the expression of the recombinant antigen(s).

Polypeptides comprising truncated Plasmodium amino acid sequences encoding at least one Plasmodium epitope can be identified in a number of ways. For example, the entire viral protein sequence can be screened by preparing a series of short peptides that together span the entire protein sequence. By starting with, for example, 100mer polypeptides, it would be routine to test each polypeptide for the presence of epitope(s) showing a desired reactivity, and then testing progressively smaller and overlapping fragments from an identified 100mer to map the epitope of interest. Screening such peptides in an immunoassay is within the skill of the art. It is also known to carry out a computer analysis of a protein sequence to identify potential epitopes, and then prepare oligopeptides comprising the identified regions for screening. However, it is appreciated by those of skill in the art that such computer analysis antigenicity does not always identify an epitope that actually exists, and can also incorrectly identify a region of the protein as containing an epitope. Methods of epitope mapping are known in the art. (See, for example, Geysen, H.M. et al., Molecular Immunology 23:709-715 (1986); Geysen, H.M. et al., Proc. Natl. Acad. Sci. USA 81:3998-4002.)

The immunogenicity of the epitopes of Plasmodium may also be enhanced by preparing them assembled with particle forming proteins. Polypeptides that are capable of forming particles when expressed in a prokaryotic system are known in the art. In preferred embodiments, a sufficient region

of the HBV core antigen is used to enable particle formation. For example, it is known that removal of the arginine rich carboxy-terminus from core does not affect particle formation. Core particles elicit both T-cell dependant and T-cell independant antibody responses, as well as a strong cellular response. (Millich D.R. and A. McLachlan, Science 234:1398 (1986); Millich, D.R. et al., J. Immunol. 139:1223 (1987); and Millich, D.R. et al., Nature 329:547 (1987).) Therefore, when the immunogenic polypeptide expressed in Salmonella is to be used in vaccine preparations, it would be desirable to include core epitopes that are responsible for one or more of the T-cell responses.

Preferably, the immunodominant antibody recognition sites comprising the amino acid repeat sequences of the CS proteins of Plasmodium are utilized in the expressed polypeptide. In P. berghei, the CS repeat sequence has been determined to be $(DP_4NPN)_3$, and in P. falciparum the CS repeat sequence has been determined to be $(NANP)_4$. These repeat sequences are capable of eliciting an immune response when incorporated into an internal insertion site of the HBcAg protein. Oligonucleotides coding for these amino acid repeat sequences have been produced synthetically and are presented in Figure 1. As shown in Figure 1, the nucleotide sequence designated $(NANP)_4$ 1 and coding for the amino acid sequence $(NANP)_4$ is the sense oligonucleotide for the P. falciparum CS repeat sequence and the nucleotide sequence designated $(NANP)_4$ 2 is the oligonucleotide complementary to $(NANP)_4$ 1. Likewise, the nucleotide sequence designated $(DP_4NPN)_3$ 1 and coding for the amino acid sequence $(DP_4NPN)_3$ is the sense oligonucleotide for the P. berghei CS repeat sequence and the nucleotide sequence designated $(DP_4NPN)_3$ 2 is the oligonucleotide complementary to $(DP_4NPN)_3$ 1.

Most preferably, the desired CS repeat sequence is inserted into an HBc core sequence to produce an HBc/CS

repeat hybrid or fusion protein. The CS repeat sequence is preferably inserted between an HBc fragment containing amino acids 1-75 and an HBc fragment containing amino acids 81-156. In addition, a fragment of the Hepatitis B pre-S(2) sequence (amino acids 133-143) is preferably fused to the carboxy terminal end of the HBc/CS hybrid for use as a marker and to verify the expression of the hybrid protein. A disgram of the structure of the HBc/CS repeat hybrid expression product of pC75CS2 (P. falciparum) and pC75CS1 (P. berghei) are presented in Figure 2.

The portions of the DNA constructs encoding the desired Plasmodium antigenic regions are then ligated to control regions that govern their expression in Salmonella and/or E. coli. Typically, the the vectors containing the CS repeats in the HBc core protein are inserted into a suitable E. coli host to verify expression of the hybrid protein. Additionally, the sequences of the vectors are verified by dideoxy DNA sequencing. The vectors can then be moved into a desired Salmonella strain by standard methodology. Generally, expression control sequences for prokaryotes include promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Commonly used prokaryotic control sequences include the Beta-lactamase (penicillinase) and lactose promoter systems (Chang et al. (1977)), the tryptophan (trp) promoter system (Goeddel et al. (1980)) and the lambda-derived P_L promoter and N gene ribosome binding site (Shimatake et al. (1981)) and the hybrid tac promoter (De Boer et al. (1983)) derived from sequences of the trp and

lac UV5 promoters. Corresponding control sequences are known for various Salmonella spp.

Recombinant polynucleotides encoding the desired Plasmodium immunogenic epitopes (also referred to as "antigenic determinants") are inserted into the Salmonella host cells by transformation. Transformation may be by any known method for introducing polynucleotides into host cells, including, for example, packaging the polynucleotide in a virus and transducing the host cell with the virus, and by direct uptake of the polynucleotide. A particularly suitable method for direct uptake is electroporation, and example of which is described infra.

The recombinant polynucleotide encoding one or more immunogenic determinants of Plasmodium are preferably in the form of a vector, particularly one comprised of the asd gene (as discussed below). Vector construction employs techniques which are known in the art. Site-specific DNA cleavage is performed by treating with suitable restriction enzymes under conditions which generally are specified by the manufacturer of these commercially available enzymes. In general, about 1 microgram of plasmid or DNA sequence is cleaved by 1 unit of enzyme in about 20 microliters buffer solution by incubation of 1-2 hr at 37°C. After incubation with the restriction enzyme, protein is removed by phenol/chloroform extraction and the DNA recovered by precipitation with ethanol. The cleaved fragments may be separated using polyacrylamide or agarose gel electrophoresis techniques, according to the general procedures found in Methods in Enzymology (1980) 65:499-560.

Sticky ended cleavage fragments may be blunt ended using E. coli DNA polymerase I (Klenow) in the presence of the appropriate deoxynucleotide triphosphates (dNTPs) present in the mixture. Treatment with S1 nuclease may also be used, resulting in the hydrolysis of any single stranded DNA portions.

Ligations are carried out using standard buffer and temperature conditions using T4 DNA ligase and ATP; sticky and ligations require less ATP and less ligase than blunt and ligations. When vector fragments are used as part of a ligation mixture, the vector fragment is often treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase to remove the 5'-phosphate and thus prevent religation of the vector; alternatively, restriction enzyme digestion of unwanted fragments can be used to prevent ligation.

Ligation mixtures are transformed into suitable cloning hosts, such as E. coli, and successful transformants selected by, for example, antibiotic resistance, and screened for the correct construction.

The desired recombinant DNA sequences may be synthesized by synthetic methods. Synthetic oligonucleotides may be prepared using an automated oligonucleotide synthesizer as described by Warner, DNA 3:401 (1984). If desired the synthetic strands may be labeled with ^{32}P by treatment with polynucleotide kinase in the presence of ^{32}P-ATP, using standard conditions for the reaction.

DNA sequences, including those isolated from Plasmodium, may be modified by known techniques, including, for example, site directed mutagenesis as described by Zoller, Nucleic Acids Res. 10:6487 (1982). Briefly, the DNA to be modified is packaged into phage as a single stranded sequence, and converted to a double stranded DNA with DNA polymerase using, as a primer, a synthetic oligonucleotide complementary to the portion of the DNA to be modified, and having the desired modification included in its own sequence. The resulting double stranded DNA is transformed into a phage supporting host bacterium. Cultures of the transformed bacteria, which contain replications of each strand of the phage, are plated in agar to obtain plaques. Theoretically, 50% of the new

plaques contain phage having the mutated sequence, and the remaining 50% have the original sequence. Replicates of the plaques are hybridized to labeled synthetic probe at temperatures and conditions which permit hybridization with the correct strand, but not with the unmodified sequence. The sequences which have been identified by hybridization are recovered and cloned.

DNA libraries may be probed using the procedure of Grunstein and Hogness, Proc. Natl. Acad. Sci. USA 73:3961 (1975). Briefly, in this procedure, the DNA to be probed is immobilized on nitrocellulose filters, denatured, and prehybridized with a buffer containing 0-50% formamide, 0.75 M NaCl, 75 mM Na citrate, 0.03% (wt/v) each of bovine serum albumin, polyvinyl pyrrolidone, and Ficoll, 50 mM Na Phosphate (pH 6.5), 0.1% SDS, and 100 micrograms/ml carrier denatured DNA. The percentage of formamide in the buffer, as well as the time and temperature conditions of the prehybridization and subsequent hybridization steps depends on the stringency required. Oligomeric probes which require lower stringency conditions are generally used with low percentages of formamide, lower temperatures, and longer hybridization times. Probes containing more than 30 or 40 nucleotides such as those derived from cDNA or genomic sequences generally employ higher temperatures, e.g., about 40-42°C, and a high percentage, e.g., 50%, formamide. Following prehybridization, 5'-^{32}P-labeled oligonucleotide probe to detect a sequence encoding a Plasmodium epitope is added to the buffer, and the filters are incubated in this mixture under hybridization conditions. After washing, the treated filters are subjected to autoradiography to show the location of the hybridized probe; DNA in corresponding locations on the original agar plates is used as the source of the desired DNA.

For routine vector constructions, ligation mixtures are transformed into E. coli strain HB101 or other suitable

host, and successful transformants selected by antibiotic resistance or other markers. Plasmids from the transformants are then prepared according to the method of Clewell et al. (1969), usually following chloramphenicol amplification (Clewell (1972)). The DNA is isolated and analyzed, usually by restriction enzyme analysis and/or sequencing. Sequencing may be by the dideoxy method of Sanger et al. Proc. Natl. Acad. Sci. USA 74:5463 (1977), as further described by Messing et al., Nucleic Acids Res. 9:309 (1981), or by the method of Maxam et al. (1980). Problems with band compression, which are sometimes observed in GC rich regions, were overcome by use of T-deazoguanosine according to Barr et al. (1986).

An enzyme-linked immunosorbent assay (ELISA) can be used to measure either antigen or antibody concentrations. This method depends upon conjugation of an enzyme to either an antigen or an antibody, and uses the bound enzyme activity as a quantitative label. To measure antibody, the known antigen is fixed to a solid phase (e.g., a microplate or plastic cup), incubated with test serum dilutions, washed, incubated with anti-immunoglobulin labeled with an enzyme, and washed again. Enzymes suitable for labeling are known in the art, and include, for example, horseradish peroxidase. Enzyme activity bound to the solid phase is measured by adding the specific substrate, and determining product formation or substrate utilization colorimetrically. The enzyme activity bound is a direct function of the amount of antibody bound.

To measure antigen, a known specific antibody is fixed to the solid phase, the test material containing antigen is added, after an incubation the solid phase is washed, and a second enzyme-labeled antibody is added. After washing, substrate is added, and enzyme activity is estimated colorimetrically, and related to antigen concentration.

When the Salmonella cells that contain the DNA construct or vector comprised of the desired Plasmodium antigenic determinant(s) are to be used in preparation of a vaccine, they ideally have a number of features. First, the cells should be completely avirulent and highly immunogenic. This requires a balance that is often difficult to achieve especially because of genetic diversity in the immunized population and significant differences in diet and hygiene between individuals. Second, at least in relation to avirulent Salmonella, it must retain its ability to colonize the intestine and GALT without causing disease or impairment of normal host physiology and growth. Third, it should have two or more attenuating mutations, preferably deletion mutations to preclude loss of the traits by reversion or gene transfer. This latter feature increases the safety of the attenuated vaccine, and is a particular consideration in human vaccines. Fourth, the attenuating phenotype should be unaffected by anything supplied in the diet or by the host individual. If the immunizing microorganism is used as a carrier microbe, the system should provide stable (or preferably high level) expression of cloned genes in the immunized individual.

Thus, in one form of this embodiment of the invention, the Salmonella strain contains at least two mutations. The second mutation increases significantly the probability that the microorganism will not revert to wild-type virulence if a reversion occurs in the first mutant gene. These mutations may be in, for example, genes which, when mutated or deleted, cause a loss of virulence (e.g., plasmid cured strains), cause the strain to be auxotrophic, cause an alteration in the utilization or synthesis of carbohydrates, or are defective in global gene expression. Examples of the latter are the cya crp Salmonella mutants described in commonly owned U.S. Serial No. 785,748, filed November 7, 1991, (some of which are also described in

Tacket, C.O. et al., Infection and Immunity 60:536-541 (1992)), and the phoP mutants described in commonly owned U.S. Serial No. 07/331,979. Contemplated as within the scope of this embodiment are microorganisms, particularly Salmonella, which contain two or more mutations of the type described above, as long as the microorganisms maintain their avirulence and immunogenicity.

Table 1. Mutations rendering Salmonella avirulent

Gene	Mutant phenotype	Reference
pab	requirement for pABA	BACON et al., 1950, 1951
asp[a]	requirement for aspartic acid	BROWN and STOCKER, 1987 BACON et al., 1950, 1951; KELLY AND CURTISS, unpublished
his[a]	requirement for histidine	BACON et al., 1950, 1951; FIELDS et al., 1986
cys[a] pur	requirement for cysteine requirement for purines	BACON et al., 1950, 1951 BACON et al., 1950, 1951; MCFARLAND and STOCKER, 1987; FIELDS et al., 1986
aroA	requirement for aromatic amino acids, pABA and dihydroxybenzoic acid	BOISETT and STOCKER, 1981; DOUCAN et al., 1987b
ard	requirement for threonine, methionine, and diaminopimelic acid	CURTISS, 1985
dap nirA nirB cmlA nmC	requirement for diaminopimelic acid requirement for adenine requirement for hypoxanthine and thiamine requirement for quinolinic acid requirement for nicotinic acid or nicotinamide mononucleotide	CLARKE and GYLES, 1987 BROWN and STOCKER, 1987 EDWARDS and STOCKER, 1988 WILSON and STOCKER, 1988 WILSON and STOCKER, 1988
ilv[a]	requirement for isoleucine and valine	KELLY and CURTISS (unpublished)
val[a]	requirement for valine	KELLY and CURTISS (unpublished)
Str[d]	streptomycin-dependent	REITMAN 1967

[a]Only some mutants of these types are avirulent and the avirulent mutants have not been investigated for immunogenicity.

Table 1. Mutations rendering Salmonella avirulent (Continued)

Gene	Mutant phenotype	Reference
galE	renders cells reversibly rough	CROMARTIE and FRASER, 1971, 1975; ROHR et al., 1987
Alpha3-1,cral	inability to transport and phosphorylate a number of carbohydrates and to regulate cell metabolism	CURTISS and KELLY, (unpublished)
pmi	renders cells reversibly rough	KELLY and CURTISS (unpublished)
Ts	decrease cell proliferation at 37°C	OSTA et al., 1987;
CYA	inefficient transport and use of carbohydrates and amino acids and inability to synthesize cell surface structures	CURTISS and KELLY, 1987
CRP	inefficient transport and use of carbohydrates and amino acids and inability to synthesize cell surface structures	CURTISS and KELLY, 1987

In another embodiment of the invention, the vaccines are comprised of microorganisms with a mutation in *phoP* or its equivalent gene, and the microorganisms are "carriers" which contain a recombinant gene(s) encoding a heterologous polypeptide(s) so that the expression product(s) of the recombinant gene(s) is delivered to the colonization site in the individual treated with the vaccine. The recombinant gene in the carrier microorganisms would encode an antigen of a fungal, bacterial, parasitic, or viral disease agent, or an allergen. Live vaccines are particularly useful where local immunity is important and might be a first line of defense. The requirement that the carrier microbe be avirulent is met by the *phoP* mutation in the microbe. However, also contemplated as within the scope of this embodiment are microorganisms, particularly *Salmonella*, which have at least one additional mutation to lessen the probability of reversion of the microorganism to wild-type virulence. Examples of these types of mutations are described supra.

In the case of carrier microorganisms, it may also be desirable to genetically engineer the PhoP$^-$ type microorganisms so that they are "balanced lethals" in which non-expression of a recombinant heterologous polypeptide(s) is linked to death of the microorganism.

"Balanced lethal" mutants of this type are characterized by a lack of a functioning native chromosomal gene encoding an enzyme which is essential for cell survival, preferably an enzyme which catalyzes a step in the biosynthesis of an essential cell wall structural component, and even more preferably a gene encoding beta-aspartic semialdehyde dehydrogenase (asd). The mutants, however, contain a first recombinant gene encoding an enzyme which is a functional replacement for the native enzyme, wherein the first recombinant gene cannot replace the defective chromosomal gene. The first recombinant gene is structurally linked to a second recombinant gene

encoding a desired product. Loss of the first recombinant gene causes the cells to die, by lysis in the cases of loss of asd, when the cells are in an environment where a product due to the expression of the first recombinant gene is absent. Methods of preparing these types of "balanced lethal" mutants are disclosed in U.S.S.N. 251,304, filed October 3, 1988, which is commonly owned by the herein assignee, and which is incorporated herein by reference.

Methods of protecting against virulent infections with vaccines employing transposon-induced avirulent mutants of virulent agents in which the impairment leading to avirulence cannot be repaired by diet or by anything supplied by an animal host have been developed. For example, a method for creating an avirulent microbe by the introduction of deletion mutations in the adenylate cyclase gene (cya) and the cyclic AMP receptor protein gen (crp) of Salmonella spp. is described in EPO Pub. No. 315,682 (published 17 May 1989), and PCT Pub. No. WO 88/09669 (published 15 December 1988).

Introduction of the mutations into cya and crp of various Salmonella strains can be accomplished by use of transposons, to transfer the mutations from one Salmonella strains into another. Transposons can be added to a bacterial chromosome at many points. The characteristics of transposon insertion and deletion have been reviewed in Kleckner et al. (1977), J. Mol. Biol. 116:125. For example, the transposon Tn10, which confers resistance to tetracycline (and sensitivity to fusaric acid) can be used to create Δcya and Δcrp mutations in a variety of bacterial species, including, for example, E. coli and S. typhimurium. Methods for the creation and detection of these mutants in S. typhimurium are described in EPO Pub. No. 315,682. Utilizing Tn10, these mutations can be transposed into various isolates of Salmonella, preferably those which are highly pathogenic.

Once rendered avirulent by the introduction of the Δcya and/or Δcrp mutations, the microbes can serve as an immunogenic component of a vaccine to induce immunity against the microbe.

In another embodiment of the invention, the Salmonella which are cya mutants and/or crp mutants are further mutated, preferably by a deletion, in a gene adjacent to the crp gene which governs virulence of Salmonella. Mutation in this gene, the cdt gene, diminishes the ability of the bacteria to effectively colonize deep tissues, e.g., the spleen. When a plasmid having the crp' gene is placed in a strain with the Δ(crp-cdt), it retains its avirulence and immunogenicity thus having a phenotype similar to cya and crp mutants. Mutants with the Δ(crp-cdt) mutation containing a crp' gene on a plasmid retain the normal ability to colonize the intestinal tract and GALT, but have a diminished ability to colonize deeper tissues. The original Δ(crp-cdt) mutation as isolated in χ3622 also deleted the argD and cysG genes imposing requirements for arginine and cystaine for growth; this mutant allele has been named Δ(crp-cysG)-10. A second mutant containing a shorter deletion was isolated that did not impose an arginine requirement; it is present in χ3931 and has been named (crp-cysG)-14. Mutations in cdt in Salmonella can be either created directly, or can be introduced via transposition from another Salmonella strains such as those shown in the Examples. In addition, the cdt mutation can be created in other strains of Salmonella using techniques known in the art, and phenotypic selection using the characteristics described herein; these mutants in S. typhimurium are described in EPO Pub. No. 315,682. Utilizing Tn10, these mutations can be transposed into various isolates of Salmonella, preferably those which are highly pathogenic.

Another type of mutation that may be used to create avirulence is a mutation in phoP. The phoP gene and its

equivalents are of a type which have "global regulation of pathogenicity", i.e., they coordinately regulate a number of genes including those that encode bacterial virulence factors. It regulates the expression of virulence genes in a fashion which may be similar to that of toxR of Vibrio cholerae or vir of Bordatella pertussis. The toxR gene is discussed in Miller and Mekalanos (1984), and Taylor et al. (1987); the vir gene is discussed in Stibitz et al. (1988). Consistent with this is the suggestion by Fields et al. (1989) that the phoP product regulates the expression of genes that allow a pathogenic microorganism to survive within macrophages, and to be insensitive to defensins, which are macrophage cationic proteins with bactericidal activity. Fields et al. (1989); Miller at al. (1989). In Salmonella, the phoP gene product also controls the expression of non-specific acid phosphatase from the phoN gene.

Some characteristics of phoP-type mutant strains are exemplified by those of the immunogenic phoP mutants of S. typhimurium. These avirulent mutants are able to establish an infection of the Peyer's patches of orally infected animals for a sufficient length of time to give rise to an immune response, but are very inefficient at reaching the spleens. The phoP mutants exhibit similar capability as the pathogenic parental strains to attach to and invade tissue culture cells which are indicators for virulence of the strain. The identity of these indicator cells are known by those of skill in the art; for example, pathogenic strains of Salmonella, including S. typhimurium, invade a variety of cells in culture, such as Henle 407, Hela, Hep-2, CHO, and MDCK cells. In addition, the Salmonella mutant strains maintain parental motility, type 1 pili, and have a lipopolysaccharide (LPS) composition similar to that of the parent strains. Moreover, the phenotype of the mutant strains is stable. Methods of determining these latter characteristics are known to those of skill in the art. It

is contemplated, however, that strains carrying the phoP mutation may have their phenotypes altered by further mutations in genes other than phoP. Strains which include mutations in addition to the phoP mutation are contemplated, and are within the scope of the invention.

A further, and significant characteristic of phoP mutants results from the control of phoP over the structural gene for phosphatase, for example, non-specific acid phosphatase in Salmonella. As exemplified in Salmonella, generally, phoP-type mutants lack non-specific acid phosphatase activity. However, this lack of phosphatase activity can be overcome by a second mutation which most likely removes the expression of the structural gene for phosphatase from the control of the phoP-type gene. Thus, mutants of phoP can be obtained which maintain their avirulence, but which are Pho$^+$ in phenotype, and produce phosphatase. Thus, inability to produce phosphatase, per se, is not responsible for the avirulence of phoP mutants.

Strains carrying mutations in phoP or its equivalent gene, particularly desirable deletion mutations, can be generated by techniques utilizing transposons. Transposons can be added to a bacterial chromosome at many points. The characteristics of transposon insertion and deletion have been reviewed in Kleckner (1977). For example, the transposon Tn10, which confers resistance to tetracycline (and sensitivity to fusaric acid) can be used to create phoP mutants in a variety of bacterial species, including, for example, E. coli and a diversity of species of Salmonella, for example, S. typhimurium, S. typhi, S. enteritis, S. dublin, S. gallinarium, S. pylorum, S. arizona, and S. choleraesuis. The isolation of mutants of other organisms which contain a deletion mutation in an equivalent to a phoP gene may be achieved with transposon mutagenesis (e.g., using Tn5, Tn10, Tn916, Tn917, or other transposons known in the art) to cause the deletion in the

virulent strain, and screening for a Pho⁻ phenotype using a substrate for non-specified/acid phosphatases (e.g., 4-bromo-3-chloro-2-indolyl phosphate, or alpha-napthyl phosphate). In the event that the microorganism contains phosphatases which are not regulated by phoP or its equivalent gene, the starting strains for transposon mutagenesis must contain mutations to inactivate these phosphatases. Methods to prepare phoP mutant strains are described in commonly owned application, U.S. Serial No. 07/331,970.

There are many methods for preparing phoP mutants. In one method, insertion of Tn10 adjacent to the phoP gene is selected in a phoP mutant of S. typhimurium LT-2 by propagating the transducing phage P22 HT int on a Tn10 library in the LT-2 strain X3000 (see USSN 251,304) and selecting on Neidhardt medium with 12 units tetracycline/ml and 40 micrograms/ml 5-Bromo-4-Chloro-3 indolyl phosphate (BCIP) as the sole source of phosphate. Rare transductants that grow will most likely have Tn10 closely linked to the wild-type phoP⁺ gene. Selection of fusaric acid resistant derivatives of a number of Tn10 transductants and plating on media with BCIP should reveal delta-phoP mutations in those cases in which the Tn10 is close enough to phoP such that deletion of the DNA between the Tn10 insertions can be conveniently used to move the delta-phoP mutations to other strains by standard methods (Kleckner 1977, and U.S. Serial No. 251,304, which is owned by the herein assignee, and which is incorporated herein by reference).

Still another means of generating phoP mutations makes use of an auxotrophic mutation closely linked to the S. typhimurium phoP gene. The purB gene has such properties. A purB S. typhimurium LT-2 mutant is transduced to PurB⁺ using a P22 HT int lysate propagated on the Tn10 library referred to above and Tc^r PhoP⁻ PurB⁺ transductants are selected and identified on Neidhardt medium devoid of adenine and containing tetracycline and

BCIP. The desired mutants will have Tn10 inserted into the phoP gene (i.e., phoP::Tn10). Selection for fusaric acid resistance will generate tetracycline-sensitive delta-phoP mutations.

The delta-phoP mutation isolated in S. typhimurium LT-2 can be transduced to other Salmonella strains by using a Tn10 insertion linked to the delta-phoP::Tn10. In either case, transductants are selected for resistance to tetracycline. If the desired highly virulent Salmonella strain to be rendered avirulent by introducing a phoP mutation is sensitive to P22, one can propagate P22 HT int on either the delta-phoP strain with the linked Tn10 or on the phoP::Tn10 mutants and use the lysate to transduce the virulent Salmonella to tetracycline resistance. The Tn10 adjacent to the delta-phoP mutation or inserted into phoP can be removed by selecting for fusaric acid resistance. In the case of the phoP::Tn10 mutant a delta-phoP mutation will be generated. If the desired highly virulent Salmonella strain to be rendered avirulent by introducing a phoP mutation is resistant to P22, one can use another transducing phage such as P1L4, which will generally only efficiently infect Salmonella strains that are rough. In this case a galE mutation can be introduced into the S. typhimurium LT-2 delta-phoP or phoP::Tn10 mutants either by transduction or by selection for resistance to 2-deoxygalactose (USSN 251,304). Growth of galE mutants in the absence of galactose renders them rough and sensitive to P1L4 permitting the propagation of a transducing lysate. galE mutants of the virulent Salmonella recipient strain will also have to be selected using 2-deoxygalactose. Transduction of these galE recipients using P1L4 propagated on the galE delta-phoP with the linked Tn10 or the galE phoP::Tn10 strain can be achieved by plating for transductants on medium with tetracyclines and containing BCIP to identify phoP⁻ transductants. Selection for fusaric acid resistance will eliminate Tn10 and in the case of the

phoP::Tn10 mutant generate a delta-phoP mutation. The galE mutation can then be removed by P1L4 mediated transduction using P1L4 propagated on a galE⁻ S. typhimurium LT-2 strain that is rough due to a mutation in a gene other than galE. Such mutants are well known to those knowledgeable in the field (see Sanderson and Roth).

It should be obvious that recombinant DNA techniques can also be used to generate phoP mutations in various pathogenic bacteria. This can be accomplished using gene cloning and DNA hybridization technologies, restriction enzyme site mapping, generation of deletions by restriction enzyme cutting of cloned phoP sequences, and by allele replacement recombination to introduce the delta-phoP defect into a selected bacterial pathogen.

Methods of preparing organisms, particularly Salmonella, which can function as carrier bacteria are discussed in WO 89/03427 (published 20 April 1989), and in U.S. Serial No. 07/251,304, filed 3 October 1988, which is commonly owned. Both of these references are incorporated herein by reference. Generally, the Salmonella are treated to cause a mutation in a chromosomal gene which encodes an enzyme that is essential for cell survival, wherein this enzyme catalyzes a step in the biosynthesis of an essential cell wall structural component. An extrachromosomal genetic element, for example, a recombinant vector, is introduced into the mutant cell. This genetic element contains a first recombinant gene which encodes an enzyme which is a functional replacement for the native enzyme, but the first recombinant gene cannot replace the defective chromosomal gene. The first recombinant gene is structurally linked to a second recombinant gene encoding a polypeptide comprised of one or more immunogenic epitopes of HBV, which is to be expressed in the carrier microorganism. Loss of the first recombinant gene causes the cells to lyse when the cells are in an environment

where a product due to the expression of the first recombinant gene is absent.

A number of genes which encode enzymes essential for cell survival, which catalyze a step in the biosynthesis of an essential cell wall structural component, are known in the art, for e.g., aspartate semialdehyde dehydrogenase (Asd), which is encoded by the asd gene. Balanced lethal mutants of this type are described in Galan et al., Gene 94:29-35 (1990). A method for introducing a deletion mutation in the asd gene of Salmonella utilizing transposon mutagenesis is described in U.S. Serial No. 785,748. Also described therein, is the construction of a genetic element which carries the functional replacement for the asd gene, linked to a gene encoding an antigen which is to be expressed in the avirulent Salmonella carrier.

Administration of a live vaccine of the type disclosed above to an individual may be by any known or standard technique. These include oral ingestion, gastric intubation, or broncho-nasal-ocular spraying. All of these methods allow the live vaccine to easily reach the GALT or BALT cells and induce antibody formation and are the preferred methods of administration. Other methods of administration, such as intravenous injection, that allow the carrier microbe to reach the individual's blood stream may be acceptable. Intravenous, intramuscular or intramammary injection are also acceptable with other embodiments of the invention, as is described later.

Since preferred methods of administration are oral ingestion, aerosol spray and gastric intubation, preferred carrier microbes are those that belong to species that attach to, invade and persist in any of the lymphoepithelial structures of the intestines or of the bronchi of the animal being vaccinated. These strains are preferred to be avirulent derivatives of enteropathogenic strains produced by genetic manipulation of enteropathogenic strains. Strains that attach to, invade

and persist in Peyer's patches and thus directly stimulate production of IgA are most preferred. In animals these include specific strains of Salmonella, and Salmonella-E. coli hybrids that home to the Peyer's patches.

The dosages required will vary with the antigenicity of the gene product and need only be an amount sufficient to induce an immune response typical of existing vaccines. Routine experimentation will easily establish the required amount. Multiple dosages are used as needed to provide the desired level of protection.

The pharmaceutical carrier or excipient in which the vaccine is suspended or dissolved may be any solvent or solid or encapsulated in a material that is non-toxic to the inoculated animal and compatible with the carrier organism or antigenic gene product. Suitable pharmaceutical carriers are known in the art, and for example, include liquid carriers, such as normal saline and other non-toxic salts at or near physiological concentrations, and solid carriers, such as talc or sucrose and which can also be incorporated into feed for farm animals. Adjuvants may be added to enhance the antigenicity if desired. When used for administering via the bronchial tubes, the vaccine is preferably presented in the form of an aerosol. Suitable pharmaceutical carriers and adjuvants and the preparation of dosage forms are described in, for example, Remington's Pharmaceutical Sciences, 17th Edition, (Gennaro, Ed., Mack Publishing Co., Easton, Pennsylvania, 1985).

Immunization with a pathogen-derived gene product can also be used in conjunction with prior immunization with the avirulent derivative of a pathogenic microorganism acting as a carrier to express the gene product specified by a recombinant gene from a pathogen. Such parenteral immunization can serve as a booster to enhance expression of the secretory immune response once the secretory immune system to that pathogen-derived gene product has been

primed by immunization with the carrier microbe expressing the pathogen-derived gene product to stimulate the lymphoid cells of the GALT or BALT. The enhanced response is known as a secondary, booster, or anamnestic response and results in prolonged immune protection of the host. Booster immunizations may be repeated numerous times with beneficial results.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

EXAMPLES

Example 1

This example describes the isolation of avirulent microbes by the introduction of deletion mutations affecting cAMP synthesis and utilization and the identification of strains with mutations conferring stability of phenotype, complete avirulence and high immunogenicity.

Bacterial strains. The Escherichia coli and Salmonella typhimurium strains used are listed in Table 2.A. and B. They were maintained as frozen cultures suspended in 1% Bacto-peptone containing 5% glycerol and fast-frozen in dry ice-ethanol for storage in duplicate at -70°C and also suspended in 1% Bacto-peptone containing 50% glycerol for storage at -20°C for routine use.

Media. Complex media for routine cultivation were L broth (Lennox, Virology 1:190-206, (1965)) and Luria broth (Luria and Burrous, J. Bacteriol. 74:461-476 (1957)). Difco agar was added to Luria broth at 1.2% for base agar and 0.65% for soft agar. Penassay agar was used for routine enumeration of bacteria. Fermentation was evaluated by supplementing MacConkey base agar or Eosin

methylene blue agar (Curtiss, Genetics 58:9-54 (1968)) with 1% final concentration of an appropriate carbohydrate.

Synthetic media were minimal liquid (ML) and minimal agar (MA) supplemented with nutrients at optimal levels as previously described (Curtiss, J. Bacteriol. 89:28-40, (1965)). Buffered saline with gelatin (BSG) (Curtiss, 1965 supra) was used routinely as a diluent.

Transduction. Bacteriophage P22HTint was routinely used for transduction using standard methods (Davis et al., "A Man. for Genet. Eng.-Adv. Bacterial Genetics". Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, (1979)). An overnight culture of the donor strain was diluted 1:20 into prewarmed Luria broth, grown for 60 minutes with shaking at 37°C and then infected with P22HTint at a multiplicity of 0.01. The infection mixture was shaken overnight for approximately 15 hours, chloroform added and allowed to shake an additional 10 min at 37°C, and the suspension centrifuged (Sorvall RC5C, SS-34 rotor, 7,000 rpm, 10 min) to remove bacterial debris. The supernatant fluid containing the phage (ca. 10^{10}/ml was stored at 4°C over chloroform. Tetracycline to a concentration of 12.5 μg/ml was used to select for transduction of Tn10 insertions and Tn10-induced mutations.

Fusaric acid selection for loss of Tn10. The media and methods described by Maloy and Nunn (J. Bacteriol. 145:1110-1112, (1981)) were used. Strains with Tn10-induced mutations were grown overnight in L broth containing 12.5 mg tetracycline/ml at 37°C to approximately 5 x 10^8 CFU/ml. Cultures were then diluted 1:40 into prewarmed L broth without tetracycline and aerated at 37°C to a titer of about 2 x 10^9 CFU/ml. Suitable numbers of cells (i.e. 10^7-10^8) diluted in BSG were plated on fusaric acid-containing medium and incubated 48 hours at 37°C. Fusaric acid-resistant isolates were purified on the same selective medium. Single isolates were picked, grown and

tested for tetracycline sensitivity on Penassay agar with and without 12.5 μg tetracycline/ml.

Mice. Female BALB/c mice (6 to 8 weeks old) (Sasco, Omaha, NB) were used for infectivity and/or immunization experiments. Animals were held for one week in a quarantined room prior to being used in experiments. Experimental mice were placed in Nalgene filter-covered cages with wire floors. Food and water were given ad libitum. The animal room was maintained at 22-23°C with a period of 12 h illumination.

Animal infectivity. The virulence of S. typhimurium strains was determined following peroral (p.o.) or intraperitoneal (i.p.) inoculation. Bacteria for inoculation in mice were grown overnight as standing cultures at 37°C in L broth. These cultures were diluted 1:50 into prewarmed L broth and aerated at 37°C for approximately 4 hours to an OD_{600} of about 0.8-1.0. The cells were concentrated 50-fold by centrifugation in a GSA rotor at 7,000 rpm for 10 min at 4°C in a Sorvall RC5C centrifuge followed by suspension in BSG. Suitable dilutions were plated on Penassay agar for titer determination and on MacConkey agar with 1% maltose to verify the Cya/Crp phenotype. For all p.o. inoculations with S. typhimurium, mice were deprived of food and water for 4 hours prior to infection. They were then given 30 ml of 10% (w/v) sodium bicarbonate using a Pipetman P200 10-15 min prior to p.o. feeding of 20 μl of S. typhimurium suspended in BSG using a Pipetman P20. Food and water were returned 30 min after oral inoculation. Morbidity and mortality of mice were observed over a 30-day period. Intraperitoneal inoculation of unfasted BALB/c mice was performed using a 26-gauge 3/8" needle to deliver 100 μl of S. typhimurium bacterial suspension diluted in BSG. Morbidity and mortality of mice were observed over a 30-day period.

Evaluation of protective immunity. In initial experiments, any mice that survived infection with any S. typhimurium mutant strain for 30 days were challenged on day 31 with 10^3-10^4 times the LD_{50} dose of wild-type mouse-virulent S. typhimurium parent strain by the p.o. route. Subsequently, groups of mice were perorally immunized with various doses of a virulent mutants and then challenged with various doses of virulent wild-type parent cells at various times after the initial immunization. Morbidity and mortality were observed throughout the experiment and for a least 30 days after challenge with the wild-type parent.

Isolation of S. typhimurium strains with Δcya-12 and Δcrp-11 mutations. The wild-type, mouse-passaged virulent S. typhimurium SL1344 strain χ3339 were genetically modified as described below, using classical genetic methods similar to those described in Curtiss and Kelly (1987). The strategy consisted of transducing the original crp-773::Tn10 mutation from PP1037 and the original cya::Tn10 mutation from PP1002 into the highly virulent and invasive S. typhimurium SL1344 strain χ3339 and screening numerous independent fusaric acid resistant, tetracycline sensitive deletion mutants for complete avirulence and highest immunogenicity in mice, as well as for greatest genotypic stability.

Transduction of the Tn10 insertions in the crp and cya genes was facilitated by first making a high-titer bacteriophage P22HTint lysate on the S. typhimurium strain PP1037 containing the crp-773::Tn10 mutation and another lysate on the S. typhimurium strain PP1002 containing the cya::Tn10 mutation. The resulting P22HTint lysates were subsequently used to infect the recipient S. typhimurium χ3339 at a multiplicity of 0.3 to transduce it to tetracycline resistance with screening for a maltose-negative phenotype. The phage-bacteria infection mixtures were incubated for 20 min at 37°C before 100 μl samples

were spread onto MacConkey agar (Difco Laboratories, Detroit, MI) containing 1% maltose (final concentration) supplemented with 12.5 μg tetracycline/ml. After approximately 26 h incubation at 37°C, a tetracycline-resistant, maltose-negative colony resulting from the P22HTint (PP1037) → χ3339 infection and a tetracycline-resistant, maltose-negative colony resulting from the P22HTint (PP1002) → χ3339 infection were picked into 0.5 ml BSG and streaked onto the same selective media. The resulting χ3339 derivatives were designated χ3604 (cya::Tn10) and χ3605 (crp-773::Tn10) (Table 2.A.).

TABLE 2. Bacterial strains

Strain number	Relevant genotype	Derivation
A. *E. coli*		
CA8445	pSD110 (*crp*$^+$ Ap^r)/Δ*crp-45* Δ*cya-06*	Schroeder and Dobrogosz, J. Bacteriol. 167:616-622 (1986).
χ6060	F' *traD16 proA*$^+$ *proB*$^+$ *lacI*^{q} Δ*lacZM15*::Tn*5*/ *araD139* Δ(*ara, leu*)-*7697* Δ*lacX74* Δ*phoA20* *galE galK recA rpsE*$_{ss}$ *rpoB thi*	Goldschmidt, Thoren-Gordon and Curtiss, J. Bacteriol. 172:3988-4001 (1990).
B. *S. typhimurium*		
798	wild-type prototroph	Received from R. Wood, NADC, Ames, IA, as a swine isolate.
#30875	wild-type prototroph	Received from P. McDonough, Cornell Univ. NY as a horse isolate.
DU8802	*zhc-1411*::Tn*10*	Sanderson and Roth, Microbiol. Rev. 42:485-532 (1988).
PP1002	*cya*::Tn*10*	Postma, Keizer and Koolwijk, J. Bacteriol. 168:1107-1111 (1986).

Strain number	Relevant genotype	Derivation
PP1037	*crp-771*::Tn*10*	Postma, Keizer and Koolwijk, supra.
SGSC452	*leu hsdLT galE trpD2 rpsL120 metE551 metA22 hsdSA hsdSB ilv*	Sanderson and Roth, 1988 supra.
TT172	*cysG*::Tn*10*	Sanderson and Roth, 1986 supra.
TT2104	*zid-62*::Tn*10*	Sanderson and Roth, supra.
χ3000	LT2-Z prototroph	Gulig and Curtiss, Infect. Immun. 55:2891-2901 (1987).
χ3140	SR-11 wild-type prototroph	Gulig and Curtiss, 1987 supra.
χ3306	SR-11 *gyrA1816*	Gulig and Curtiss, 1987 supra.
χ3385	LT-2 *hsdL6 galE496 trpB2 flaA66 his-6165 rpsL120 xyl-404 metE551 metA22 lamB*$^+$ (*E. coli*) Δ[*zia*::Tn*10*] *hsdSA29 val*	Tinge and Curtiss, J. Bacteriol. 172: in press (1990).
χ3339	SL1344 wild type *hisG rpsL*	Smith et al., Am. J. Vet. Res. 43:59-66 (1984).
χ3520	Δ*asdA1 zhf-4*::Tn*10*	ATCC53681; Asd$^-$ tetracycline-resistant derivative of χ3000.
χ3604	*hisG rpsL cya*::Tn*10*	P22HT*int*(PP1002) → χ3339 with selection for tetracycline resistance (Mal$^-$).

Strain number	Relevant genotype	Derivation
χ3605	hisG rpsL crp-771::Tn10	P22HTint(PP1037) → χ3339 with selection for tetracycline resistance (Mal⁻).
χ3615	hisG rpsL Δcya-12	Fusaric acid-resistant, tetracycline-sensitive Mal⁻ derivative of χ3604.
χ3622	hisG rpsL Δ[crp-cysG]-10	Fusaric acid-resistant, tetracycline-sensitive Mal⁻ Cys⁻ Arg⁻ derivative of χ3605.
χ3623	hisG rpsL Δcrp-11	Fusaric acid-resistant, tetracycline-sensitive Mal⁻ derivative of χ3605.
χ3670	pSD110⁺ hsdL6 galE496 trpB2 flaA66 his-6165 rpsL120 xyl-404 metE551 metA22 lamB⁺ (*E. coli*) Δ[zia::Tn10 hsdSA29 val	χ3385 transformed with pSD110 from CA8445 with selection for ampicillin resistance, Mal⁺.
χ3706	pSD110⁺ hisG rpsL Δ[crp-cysG]-10	χ3622 transformed with pSD110 from CA8445 with selection for ampicillin resistance, Mal⁺.
χ3711	hisG rpsL Δcya-12 zid-62::Tn10	P22HTint(χ3738) → χ3615 with selection for tetracycline resistance, Mal⁻.

Strain number	Relevant genotype	Derivation
χ3712	hisG rpsL Δcrp-10 zhc-1431::Tn10	P22HTint(χ3741) → χ3622 with selection for tetracycline resistance, Mal⁻, (Cys⁻, Arg⁻).
χ3722	pSD110⁺ hisG rpsL Δ[crp-cysG]-10 Δcya-12 zid-62::Tn10	P22HTint(χ3711) → χ3706 with selection for tetracycline resistance (Mal⁻).
χ3723	pSD110⁺ hisG rpsL Δ[crp-cysG]-10 Δcya-12 Δ[zid-62::Tn10]	Fusaric acid-resistant, tetracycline-sensitive, ampicillin-resistant, Mal⁻, Cys⁻, Arg⁻ derivative of χ3723.
χ3724	hisG rpsL Δ[crp-cysG]-10 Δcya-12 Δ[zid-62::Tn10]	Ampicillin-sensitive derivative of χ3723; pSD110 cured by serial passage in L broth at 37°C.
χ3730	leu hsdLT galE trpD2 rpsL120 ΔasdA1 Δ[zhf-4::Tn10] metE551 metA22 hsdSA hsdSB ilv	Asd⁻ Tc⁸ derivative of SGSC452.
χ3731	pSD110⁺ hisG rpsL crp-771::Tn10	Spleen isolate of χ3706 from BALB/c mouse.
χ3738	zid-62::Tn10	P22HTint(TT2104) → χ3000 with selection for tetracycline resistance.
χ3741	zhc-1431::Tn10	P22HTint(DU8802) → χ3000 with selection for tetracycline resistance.

Strain number	Relevant genotype	Derivation
χ3761	UK-1 wild-type prototroph	ATCC68169; Spleen isolate of ∮30875 from White leghorn chick.
χ3773	hisG rpsL Δcrp-11 zhc-1431::Tn10	P22HTint(χ3741) → χ3623 with selection for tetracycline resistance (Mal^-).
χ3774	pSD110$^+$ hisG rpsL Δcrp-11	χ3623 transformed with pSD110 from CA8445 with selection for ampicillin resistance, Mal^+.
χ3777	Δ[crp-cysG]-10 zhc-1431::Tn10	P22HTint(χ3712) → 798 with selection for tetracycline resistance, Mal^-, (Cys^-, Arg^-).
χ3779	Δ[crp-cysG]-10 Δ[zhc-1431::Tn10]	P22HTint(χ3712) → ∮30875 with selection for tetracycline resistance, Mal^-, (Cys^-, Arg^-).
χ3784	Δ[crp-cysG]-10 Δ[zhc-1431::Tn10]	Fusaric acid-resistant, tetracycline-sensitive, Mal^-, Cys^-, Arg^- derivative of χ3779.
χ3806	Δ[crp-cysG]-10 Δ[zhc-1431::Tn10]	Fusaric acid-resistant, tetracycline-sensitive, ampicillin-resistant, Mal^-, Cys^-, Arg^- derivative of χ3777.

Strain number	Relevant genotype	Derivation
χ3825	Δcrp-11 zhc-1431::Tn10	P22HTint(χ3773) → 798 with selection for tetracycline resistance, Mal^-.
χ3828	Δcrp-11 zhc-1431::Tn10	P22HTint(χ3773) → UK-1 with selection for tetracycline resistance, Mal^-.
χ3876	Δcrp-11 Δ[zhc-1431::Tn10]	Fusaric acid-resistant, tetracycline-sensitive, Mal^- derivative of χ3825.
χ3901	pSD110$^+$ Δ[crp-cysG]-10 Δ[zhc-1431::Tn10]	P22HTint(χ3670) → χ3806 with selection for ampicillin resistance, Mal^+, (Cys^-, Arg^-).
χ3902	pSD110$^+$ Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] Δcya-12 zid-62::Tn10	P22HTint(χ3711) → χ3901 with selection for tetracycline resistance, Mal^-, (Cys^-, Arg^-).
χ3910	hisG rpsL cysG::Tn10	P22HTint(TT172) → χ3339 with selection for tetracycline resistance, Cys^-.
χ3931	hisG rpsL Δ[crp-cysG]-14	Fusaric acid-resistant, tetracycline-sensitive, Mal^-, Cys^-, (Arg^+) derivative of χ3910.
χ3936	hisG rpsL Δcrp-11 Δcya-12 zid-62::Tn10	P22HTint(χ3711) → χ3774 with selection for tetracycline resistance, Mal^-.

Strain number	Relevant genotype	Derivation
χ3937	hisG rpsL Δcrp-11 Δcya-12 zid-62::Tn10	Fusaric acid-resistant, tetracycline sensitive, Mal⁻ derivative of χ3936.
χ3938	pSD110⁺ Δcrp-11 Δ(zhc-1431::Tn10)	P22HTint(χ3670) ⇒ χ3876 with selection for ampicillin resistance, Mal⁺.
χ3939	hisG rpsL Δcrp-11 Δcya-12 Δ(zid-62::Tn10)	Ampicillin-sensitive derivative of χ3937; pSD110 cured by serial passage in L broth at 37°C.
χ3945	pSD110⁺ Δ(crp-cysG)-10 Δ(zhc-1431::Tn10)	P22HTint(χ3670) ⇒ χ3784 with selection for ampicillin resisbance, Mal⁺.
χ3954	Δcrp-11 Δ(zhc-1431::Tn10)	Fusaric acid-resistant, tetracycline-sensitive, Mal⁻ derivative of χ3828.
χ3955	hisG rpsL Δ(crp-cysG)-14	P22HTint(χ3670) ⇒ χ3931 with selection for ampicillin resistance, Mal⁺, (Cys⁻, Arg⁺).
χ3956 •	pSD110⁺ Δ(crp-cysG)-10 Δ(zhc-1431::Tn10) Δcya-12 zid-61::Tn10	P22HTint(χ3711) ⇒ χ3945 with selection for tetracycline resistance, Mal⁻, Cys⁻, Arg⁻.

Strain number	Relevant genotype	Derivation
χ3957	pSD110⁺ Δ(crp-cysG)-10 Δ(zhc-1431::Tn10) Δcya-12 Δ(zid-61::Tn10)	Fusaric acid-resistant, tetracycline-sensitive, Mal⁻, Cys⁻, Arg⁻ derivative of χ3956.
χ3958	Δ(crp-cysG)-10 Δ(zhc-1431::Tn10) Δcya-12 Δ(zid-61::Tn10)	Ampicillin-sensitive derivative of χ3957; pSD110 cured by serial passage in L broth at 37°C.
χ3961	pSD110⁺ Δcrp-11 Δ(zhc-1431::Tn10)	P22HTint(χ3670) ⇒ χ3954 with selection for ampicillin resistance, Mal⁺.
χ3962	pSD110⁺ Δcrp-11 Δ(zhc-1431::Tn10) Δcya-12 zid-62::Tn10	P22HTint(χ3711) ⇒ χ3961 with selection for tetracycline resistance, Mal⁻.
χ3978	pSD110⁺ Δcrp-11 Δ(zhc-1431::Tn10) Δcya-12 zid-62::Tn10	P22HTint(χ3711) ⇒ χ3938 with selection for tetracycline resistance, Mal⁻.
χ3985	Δcya-12 Δ(zid-62::Tn10) Δcrp-11 Δ(zhc-1431::Tn10)	ATCC68166; Fusaric acid-resistant, tetracycline-sensitive, Mal⁻ derivative of χ3962 cured of pSD110.
χ4038	Δcya-12 Δ(zid-62::Tn10) Δ(crp-cysG)-10 Δ(zhc-1431::Tn10)	Fusaric acid-resistant tetracycline-sensitive Mal⁻, Cys⁻, Arg⁻ derivative of χ3902 cured of pSD110.

Strain number	Relevant genotype	Derivation
x4039	Δcya-12 Δ(zid-62::Tn10) Δ[crp-cysG]-10 Δ[zhc-1411::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Mal⁻ derivative of χ3978 cured of pSD110.
x4063	SR-11 arg::Tn10	P22HTint(Tn10 library) ⇒ χ3306 with selection for tetracycline resistance, Arg⁻.
x4071	SR-11 arg::Tn10	P22HTint(Tn10 library) ⇒ χ3306 with selection for tetracycline resistance, Arg⁻.
x4246	Δ[crp-cysG]-10 zhc-1411::Tn10	P22HTint(χ3712) ⇒ 798 with selection for tetracycline resistance, Mal⁻, (Cys⁻ Arg⁻).
x4247	pSD110⁺ Δ[crp-cysG]-10 zhc-1411::Tn10	P22HTint(χ3670) ⇒ χ4246 with selection for ampicillin resistance, Mal⁺, (Cys⁻ Arg⁻).
x4248	Δ[crp-cysG]-10 zhc-1411::Tn10	P22HTint(χ3712) ⇒ ATCC68169 (UK-1) with selection for tetracycline resistance, Mal⁻, (Cys⁻ Arg⁻).
x4262	pSD110⁺ Δ[crp-cysG]-10 zhc-1411::Tn10	P22HTint(χ3670) ⇒ χ4248 with selection for ampicillin resistance, Mal⁺, (Cys⁻ Arg⁻).

Strain number	Relevant genotype	Derivation
C. S. typhi		
Ty2	Type E1 Cys⁻ Trp⁻ wild type	Louis Baron, Walter Reed Army Institute of Research.
ISP1820	Type 46 Cys⁻ Trp⁻ wild type	Center for Vaccine Development, Baltimore, MD; 1983 isolate from Chilean patient.
ISP2822	Type E1 Cys⁻ Trp⁻ wild type	Center for Vaccine Development, Baltimore, MD; 1983 isolate from Chilean patient.
χ3791	Δ[crp-cysG]-10 zhc-1411::Tn10	P22HTint(χ3712) ⇒ ISP2822 with selection for tetracycline resistance (Mal⁻, Cys⁻, Arg⁻, Vi⁺).
χ3792	Δ[crp-cysG]-10 zhc-1411::Tn10	P22HTint(χ3712) ⇒ Ty2 with selection for tetracycline resistance (Mal⁻, Cys⁻, Arg⁻ Vi⁺).
χ3802	Δ[crp-cysG]-10 Δ[zhc-1411::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Mal⁻ derivative of χ3791 (Vi⁺).
χ3803	Δ[crp-cysG]-10 Δ[zhc-1411::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Mal⁻ derivative of χ3792 (Vi⁺).

Strain number	Relevant genotype	Derivation
χ3824	pSD110$^+$ Δ[crp-cysG]-10 Δ[zhc-1431::Tn10]	χ3803 electro-transformed with pSD110 from χ3670 with selection for ampicillin resistance (Mal$^+$, Cys$^-$, Arg$^-$, Vi$^+$).
χ3845	pSD110$^+$ Δ[crp-cysG]-10 Δ[zhc-1431::Tn10]	χ3802 electro-transformed with pSD110 from χ3670 with selection for ampicillin resistance (Mal$^+$, Cys$^-$, Arg$^-$, Vi$^+$).
χ3852	Δcrp-11 zhc-1431::Tn10	P22HTint(Δ3773) ⇒ ISP2822 with selection for tetracycline resistance (Mal$^-$, Vi+).
χ3853	Δcrp-11 zhc-1431::Tn10	P22HTint(χ3773) ⇒ Ty2 with selection for tetracycline resistance (Mal$^-$, Vi$^+$).
χ3877	Δcrp-11 Δ[zhc-1431::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Mal$^-$ derivative of χ3852 (Vi$^+$).
χ3878	Δcrp-11 Δ[zhc-1431::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Mal$^-$ derivative of χ3853 (Vi$^+$).
χ3879	pSD110$^+$ Δcrp-11 Δ[zhc-1431::Tn10]	P22HTint(χ3670) ⇒ Δ3877 with selection for ampicillin resistance (Mal$^+$, Vi$^+$).
χ3880	pSD110$^+$ Δcrp-11 Δ[zhc-1431::Tn10]	P22HTint(χ3670) ⇒ χ3878 with selection for ampicillin resistance (Mal$^+$, Vi$^+$).

Strain number	Relevant genotype	Derivation
χ3919	pSD110$^+$ Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] Δcya-12 zid-62::Tn10	P22HTint(χ3711) ⇒ χ3824 with selection for tetracycline resistance (Mal$^-$, Vi$^+$).
χ3920	pSD110$^+$ Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] Δcya-12 zid-62::Tn10	P22HTint(χ3711) ⇒ χ3845 with selection for tetracycline resistance (Mal$^-$, Vi$^+$).
χ3921	pSD110$^+$ Δcrp-11 Δ[zhc-1431::Tn10] Δcya-12 zid-62::Tn10	P22HTint(χ3711) ⇒ χ3879 with selection for tetracycline resistance (Mal$^-$, Vi$^+$).
χ3922	pSD110$^+$ Δcrp-11 Δ[zhc-1431::Tn10] Δcya-12 zid-62::Tn10	P22HTint(χ3711) ⇒ χ3880 with selection for tetracycline resistance (Mal-, Vi$^+$).
χ3924	Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Mal$^-$ derivative of χ3919 cured of pSD110 (Vi$^+$).
χ3925	Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Mal$^-$ derivative of χ3920 cured of pSD110 (Vi$^+$).

Strain number	Relevant genotype	Derivation
χ3926	Δcrp-11 Δ[zhc-1411::Tn10] Δcya-12 Δ[zid-62::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Mal⁻ derivative of χ3921 cured of pSD110 (Vi⁺).
χ3927	Δcrp-11 Δ[zhc-1411::Tn10] Δcya-12 Δ[zid-62::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Mal⁻ derivative of χ3922 cured of pSD110 (Vi⁺).
χ3940	Δ[crp-cysG]-10 Δ[zhc-1411::Tn10] Δcya-12 Δ[zid-62::Tn10]	Flagella-positive, motile derivative of χ3925 (Vi⁺).
χ4073	Δ[crp-cysG]-10 Δ[zhc-1411::Tn10] Δcya-12 Δ[zid-62::Tn10]	Flagella-positive, motile derivative of χ3924 (Vi⁺).
χ4296	Δcrp-11 Δ[zhc-1411::Tn10] Δcya-12 Δ[zid-62::Tn10] ΔasdA1 zhf-4::Tn10	P22HTint(χ3520) ⇒ χ3927 with selection for tetracycline resistance and screening for Asd⁻, Mal⁻, Vi⁺.
χ4297	Δcrp-11 Δ[zhc-1411::Tn10] Δcya-12 Δ[zid-62::Tn10] ΔasdA1 Δ[zhf-4::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Asd⁻, Mal⁻ derivative of χ4296 (Vi⁺).
χ4298	Δcrp-11 zhc-1411::Tn10	P22HTint(χ3773) ⇒ ISP1820 with selection for tetracycline resistance (Mal⁻, Vi⁺).

Strain number	Relevant genotype	Derivation
χ4299	Δcrp-11 Δ[zhc-1411::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Mal⁻ derivative of χ4298 (Vi⁺).
χ4300	pSD110⁺ Δcrp-11 Δ[zhc-1411::Tn10]	P22HTint(χ3670) ⇒ Δ4299 with selection for ampicillin resistance (Mal⁺, Vi⁺).
χ4316	pSD110⁺ Δcrp-11 Δ[zhc-1411::Tn10] Δcya-12 zid-62::Tn10	P22HTint(χ3670) ⇒ χ4300 with selection for tetracycline resistance (Mal⁻, Vi⁺).
χ4322	Δcrp-11 Δ[zhc-1411::Tn10] Δcya-12 Δ[zid-62::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Mal⁻ derivative of χ4316 cured of pSD110 (Vi⁺).
χ4323	Δcrp-11 Δ[zhc-1411::Tn10] Δcya-12 Δ[zid-62::Tn10]	Flagella-positive, motile derivative of χ4322 (Vi⁺)
χ4324	Δ[crp-cysG]-10 zhc-1411::Tn10	P22HTint(χ3712) ⇒ ISP1820 with selection for tetracycline resistance (Mal⁻, Cys⁻, Arg⁻, Vi⁺).
χ4325	Δ[crp-cysG]-10 Δ[zhc-1411::Tn10	Fusaric acid-resistant, tetracycline-sensitive Mal⁻ derivative of χ4324 (Vi⁺).

Strain number	Relevant genotype	Derivation
χ4331	pSD110$^+$ Δ[crp-cysG]-10 Δ[zhc-1431::Tn10]	P22HTint(χ3670) ⇒ χ4325 with selection for ampicillin resistance (Mal$^+$, Vi$^+$).
χ4340	pSD110$^+$ Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] Δcva-12 zid-62::Tn10	P22HTint(χ3711) ⇒ χ4331 with selection for tetracycline resistance (Mal$^-$, Vi$^+$).
χ4345	Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] Δcva-12 Δ[zid-62::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Mal$^-$ derivative of χ4340 cured of pSD110 (Vi$^+$).
χ4346	Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] Δcva-12 Δ[zid-62::Tn10]	Flagella-positive, motile derivative of χ4345 (Vi$^+$).
χ4416	Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] ΔasdA1 zhf-4::Tn10 Δcva-12 Δ[zid-62::Tn10]	P22HTint (χ3520)→χ4346 with selection for tetracycline resistance and screening for Asd$^-$, Mal$^-$, Vi$^+$.
χ4417	[Δcrp-cysG]-10 Δ[zhc-1431::Tn10] ΔasdA1 zhf-4::Tn10 Δcva-12 Δ[zid-62::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Asd$^-$, Mal$^-$ derivative of χ4416 (Vi$^+$).

Strain number	Relevant genotype	Derivation
χ4434	Δcrp-11 Δ[zhc-1431::Tn10] ΔasdA1 zhf-4::Tn10] Δcva-12 Δ[zid-62::Tn10]	P22HTint (χ3520)→χ4323 with selection for tetracycline resistance and screening for Mal$^-$, Asd$^-$, Vi$^+$.
χ4435	Δcrp-11 Δ[zhc-1431::Tn10] ΔasdA1 Δ[zhf-4::Tn10] Δcva-12 Δ[zid-62::Tn10]	Fusaric acid-resistant, tetracycline-sensitive Mal$^-$ derivative of χ4434 (Vi$^+$).

Strains χ3604 and χ3605 were grown in L broth + 12.5 μg tetracycline/ml and 100 μl samples of each strain diluted 1:10 into buffered saline with gelatin (BSG) were spread onto 10 plates of fusaric acid-containing (FA) media (Maloy and Nunn, 1981). The plates were incubated approximately 36 hours at 37°C. Five fusaric acid-resistant colonies from each plate were picked into 0.5 ml BSG and purified on FA media. Purified fusaric acid-resistant colonies were picked into L broth and grown at 37°C to turbidity and checked for loss of Tn*10* (tetracycline sensitivity). One tetracycline-sensitive derivative was selected from each of the ten platings on FA media and characterized for complete LPS (by P22HT*int* sensitivity), auxotrophy or prototrophy, stability of the gene deletion, and reversion to tetracycline resistance. This procedure resulted in ten independently isolated Δ*cya* mutants from χ3605 and ten independently isolated Δ*crp* mutants from χ3605.

Genetic stability of avirulent mutants.

Strains to be orally administered as live vaccines must have complete stability with regard to both their avirulence and their immunogenic attributes. When 50-fold concentrated cultures and various dilutions (~10^9, 10^7, 10^5, 10^3 CFU/plate) of each of the ten independent Δ*cya* mutants and each of the ten independent Δ*crp* mutants were plated on minimal agar media (supplemented with 22 μg cysteine/ml and 22 μg arginine/ml) containing 0.5% maltose, melibiose, xylose, glycerol, or rhamnose that should not support their growth, revertants and mutants were not detected. One set of duplicate plates were UV-irradiated (5 joules/meter²/sec) and incubated at 37°C with illumination. Revertants and mutants were not detected after a 48 hour growth period. An investigation was also conducted as to whether tetracycline-resistant revertants/mutants could be recovered from the fusaric acid resistant Δ*cya* and Δ*crp* mutants at frequencies higher than could be observed for

the tetracycline-sensitive wild-type parental strain. In all cases, such tetracycline-resistant revertants/mutants were not observed.

Virulence and immunogenicity of Δcrp and Δcya mutants. The resulting tan Δ*crp* and tan Δ*cya* mutants were screened in BALB/c mice by peroral inoculation to determine the lowest virulence and disease symptomology as revealed by the appearance of the coat (scruffy versus smooth), appetite, and activity (high or low). Five mice per group were p.o. inoculated with ~10^9 CFU of each of the independent *cya* or *crp* deletion mutants. Animals were scored based on the above criteria and on day 30 of the experiment the survivors were challenged with 10^9 CFU of the wild-type virulent parent strain χ3339. In three of the twenty groups infected with the *cya* or *crp* deletion mutants, five of five mice survived the initial infection with the Δ*cya-12*, Δ*crp-11* and Δ*crp-10* mutants and were also completely protected against 10^4 LD_{50}s of the wild-type challenge. One group in particular, the Δ*crp-10* mutant, was unequalled in avirulence, immunogenicity and stability. After repeating these experiments, mice never appeared affected by any dose given p.o. or i.p. of the Δ*crp-10* mutant (see Example 3).

Properties of selected mutant strains. χ3615, χ3622 and χ3623 with the Δ*cya-12*, Δ*crp-10* and Δ*crp-11* mutations, respectively, were judged to be least virulent, highly immunogenic and extremely stable phenotypically and genotypically. Data on the phenotypic properties of these strains is given in Table 3. Table 4 presents data on the avirulence and immunogenicity of these strains in comparison to results with the virulent wild-type parent χ3339 and strains χ3604 and χ3605 with the *cya*::Tn*10* and *crp-773*::Tn*10* mutations, respectively. In addition to requiring histidine, which is due to the *hisG* mutation in the parental χ3339, the Δ*crp-10* mutation imposed on χ3622 requirements for the amino acids arginine and cysteine.

The bases for this observation and further analysis of the properties of the Δcrp-10 mutation are given in Example 3.

Table 2

Phenotypic characteristics of S. typhimurium Δcya and Δcrp strains

Strain and genotype	P22[a]	Carbohydrate fermentation and use[b]							Auxotrophy		
		Mal	Mtl	Ino	Srl	Rha	Mel	Gal	Glc	His	Arg	Cys
χ3339 wild type	S	+	+	+	+	+	+	+	+	-	+	+
χ3615 Δcya-12	S	-	-	-	-	-	-	+/-	+	-	+	+
χ3622 Δcrp-10	S	-	-	-	-	-	-	+/-	+	-	-	-
χ3623 Δcrp-11	S	-	-	-	-	-	-	+/-	+	-	+	+

[a]Bacteriophage P22HT*int* S=Sensitive; R=Resistant

[b]Fermentation on MacConkey Base agar media and API 20E and growth on MA + 0.5% of carbon source.

Table 4

Virulence and immunogenicity of S. typhimurium cya::Tn10, crp::Tn10 Δcya-12, Δcrp-11 and Δcrp-11 mutants in BALB/c mice

Strain number	Relevant genotype	P.O. immunization Dose (CFU)	P.O. immunization Survival live/total	Wild-type P.O. challenge Dose (CFU)	Wild-type P.O. challenge Survival live/total
χ3339	wild type	--	--	6.0×10^4	2/5
χ3604	cya::Tn10	6.2×10^8	5/5	8.8×10^8	4/5
χ3605	crp-773::Tn10	6.8×10^8	5/5	8.8×10^8	5/5
χ3615	Δcya-12	2.2×10^9	5/5	3.2×10^8	5/5
χ3622	Δcrp-10	1.5×10^9	5/5	3.2×10^8	5/5
χ3623	Δcrp-11	4.6×10^8	5/5	8.8×10^8	5/5

Example 2

This example describes the construction of avirulent microbes by the introduction of deletion mutations affecting cAMP synthesis and utilization and the characterization of strains with two deletion mutations for stability of phenotype, complete avirulence and high immunogenicity.

Bacterial strains. The Escherichia coli and Salmonella typhimurium strains used are listed in Table 2.A. and B. The maintenance and storage of these strains are as described in Example 1.

Media. Complex media for routine cultivation, enumeration and identification of bacteria are as described in Example 1.

Transduction and fusaric acid selection for loss of Tn10. The media and methods are as described in Example 1.

Animal infectivity and evaluation of protective immunity. The virulence and immunogenicity of S. typhimurium strains were determined as described in Example 1.

Construction of S. typhimurium strains with Δcya-12 and Δcrp-11 deletion mutations. The best vaccine strains in terms of efficacy are likely to result from the attenuation of highly virulent strains that display significant colonizing ability and invasiveness. The criteria for selection of these highly pathogenic S. typhimurium wild-type strains such as SL1344 (χ3339), UK-1 (χ3761) and 798 included low LD_{50} values in mouse virulence assays, antibiotic sensitivity, possession of the virulence plasmid, ease of genetic manipulation (bacteriophage P22HTint or P1 sensitivity, transformability and ease of receiving mobilized plasmids), and colicin sensitivity.

The wild-type, virulent S. typhimurium strains SL1344 (χ3339), 798 and UK-1 (χ3761) were genetically modified as described below, using classical genetic methods similar to those described in Curtiss and Kelly

(1987). The strategy consists of mobilizing deletions of crp and cya genes that have been isolated and characterized in S. typhimurium SL1344 (as described in Example 1) by placing the transposon Tn10 (encoding tetracycline resistance) nearby the Δcya-12 or Δcrp-11 mutation and transducing the linked traits into the highly virulent S. typhimurium strains UK-1 χ3761, 798 and SL1344 χ3339 via P22HTint-mediated transduction with selection for tetracycline resistance and screening for a maltose-negative phenotype. The zhc-1431::Tn10 linked to Δcrp-11 and zid-62::Tn10 linked to Δcya-12 were used for this purpose. Neither insertion alone affects the virulence of S. typhimurium.

Transduction of the gene deletions with the linked transposon was facilitated by first making a high-titer bacteriophage P22HTint lysate on the S. typhimurium strain χ3773 containing the Δcrp-11 and zhc-1431::Tn10 mutations and another lysate on the S. typhimurium strain χ3711 containing the Δcya-12 and zid-62::Tn10 mutations. The resulting P22HTint lysates were then used to transduce the genetic trains into the wild-type recipient strains χ3339, 798 and χ3761.

P22HTint propagated on S. typhimurium χ3773 (Δcrp-11 zhc-1431::Tn10) was used to transduce the virulent strains to tetracycline resistance with screening for Mal⁻. The phage-bacteria infection mixtures were incubated for 20 min at 37°C before 100 μl samples were spread onto MacConkey agar (Difco Laboratories, Detroit, MI) containing 1% maltose (final concentration) supplemented with 12.5 μg tetracycline/ml. After approximately 26 h incubation at 37°C, tetracycline resistant Mal⁻ transductants were picked and purified onto the same medium. The resulting 798 derivative was designated χ3825 and the UK-1 derivative was designated χ3828. Strains χ3773, χ3825 and χ3828 have the genotype Δcrp-11 zhc-1431::Tn10 (Table 2.B.). These strains were grown in L broth + 12.5 μg tetracycline/ml and

each were diluted 1:10 into buffered saline with gelatin (BSG), 100 μl of each were spread onto fusaric acid-containing (FA) media (Maloy and Nunn, 1981) and the plates were incubated approximately 36 h at 37°C. Fusaric acid-resistant colonies of each strain were picked into 0.5 ml BSG and purified onto FA media. Purified fusaric acid-resistant colonies were picked into L broth and grown at 37°C to turbidity and checked for loss of Tn10 (tetracycline sensitivity), presence of complete LPS and auxotrophy. The new strains were designated χ3876 (798) and χ3954 (UK-1) which both have the genotype Δcrp-11 Δ[zhc-1431::Tn10] and χ3623 (SL1344 Δcrp-11 was originally isolated as described in Example 1) (Table 2.B.).

Since the phenotype of Cya⁻ and Crp⁻ mutants are the same (Mal⁻, Stl⁻, Mtl⁻, etc.), the plasmid, pSD110, carrying the cloned crp⁺ gene and conferring ampicillin resistance (Schroeder and Dobrogosz, J. Bacteriol 167::616-622 (1986)), was used to temporarily complement the Δcrp mutation in the chromosome enabling the identification of the Δcya mutation when introduced via transduction. L broth grown cultures of χ3623, χ3876 and χ3954 were transduced with P22HTint propagated on S. typhimurium χ3670, which contains the plasmid pSD110 (Table 2.B.). Selection was made on MacConkey agar + 1% maltose + 100 μg ampicillin/ml. After 26 h, an ampicillin-resistant, Mal⁺ colony of each strain was picked and purified on MacConkey agar + 1% maltose agar + 100 μg ampicillin/ml and designated χ3938 (798) and χ3961 (UK-1) which both have the genotype Δcrp-11 Δ[zhc-1431::Tn10] pSD110⁺ and χ3774 (SL1344) which has the genotype Δcrp-11 pSD110⁺.

Strains χ3774, χ3938 and χ3961 were grown in L broth + 100 μg ampicillin/ml and were each independently transduced with P22HTint propagated on χ3711 to introduce the linked Δcya-12 and zid-62::Tn10 mutations. The transduction mixtures were plated on MacConkey agar + 1% maltose + 100 μg ampicillin/ml + 12.5 μg tetracycline/ml.

Ampicillin-resistant (pSD110$^+$), tetracycline-resistant (zid-62::Tn10), Mal$^-$ (Δcya) colonies were picked and purified on MacConkey agar + 1% maltose + 100 μg ampicillin/ml + 12.5 μg tetracycline/ml. Purified colonies were picked into L broth, grown to turbidity and the strains checked for complete LPS and auxotrophy. The resulting strains were designated χ3978 (798) and χ3962 (UK-1) which both have the genotype Δcrp-11 Δ[zhc-1431::Tn10] pSD110$^+$ Δcya-12 zid-62::Tn10 and χ3936 (SL1344) which has the genotype Δcrp-11 pSD110$^+$ Δcya-12 zid-62::Tn10. Cultures of χ3936, χ3978 and χ3962 were grown in L broth + 100 μg ampicillin/ml + 12.5 μg tetracycline/ml to turbidity, diluted 1:10 into BSG, and 100 μl samples of each culture spread onto fusaric acid-containing media and incubated approximately 36 h at 37°C. Fusaric acid-resistant colonies of each strain were picked and purified onto FA medium. Purified FA-resistant colonies were picked into L broth, grown to turbidity and then checked for loss of Tn10 (tetracycline sensitivity), complete LPS and auxotrophy. The pSD110 plasmid was usually lost spontaneously from the strains during this process to result in ampicillin sensitivity, except for the SL1344 derivative which involved two steps to eliminate pSD110. The final strains were designated χ4039 (798) and χ3985 (UK-1) which both have the genotype Δcrp-11 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10] and χ3939 (SL1344) which has the genotype Δcrp-11 Δcya-12 Δ[zid-62::Tn10] (Table 2.B.).

Genotypic and phenotypic stability of avirulent mutants. Methods for determining stability of genetic traits are as described in Example 1. All genotypic and phenotypic traits due to the Δcya Δcrp mutations were completely stable except motility. Although synthesis of functional flagella and display of motility is dependent on wild-type cya and crp gene functions, a suppressor mutation in the cfs (constitutive flagellar synthesis) gene can easily be selected to cause flagella synthesis and motility

to be independent of cya and crp gene functions. In S. typhimurium Δcya Δcrp strains, motile variants were readily selected during the strain construction process. Since immunity to flagellar antigens may be protective, motile variants of all vaccine strains were selected.

S. typhimurium group B O-antigen synthesis was confirmed by slide agglutination with antisera (Difco Laboratories, Detroit, MI) and by P22HTint bacteriophage sensitivity by the Luria soft agar overlay technique.

Fermentation of sugars and growth on various carbon sources of the double mutant strains were identical to strains with only Δcya or Δcrp as listed in Table 3. The phenotypes were as expected based on published reports of the requirement for cyclic AMP and the cyclic AMP receptor protein for catabolic activities.

At each step in the construction following selection of a fusaric acid-resistant tetracycline-sensitive derivative, an investigation as to whether tetracycline-resistant revertants/mutants could be recovered at frequencies higher than could be observed for the parental tetracycline-sensitive wild-type strain was conducted. In all cases, such tetracycline-resistant revertants/mutants were not observed.

Virulence of mutant strains for mice. Preliminary information on virulence of S. typhimurium mutant strains was obtained by infecting individual mice with 10^8 mutant cells perorally and recording morbidity and mortality. Table 5 presents data on morbidity and mortality of mice infected perorally with the S. typhimurium wild-type parent strains, and the Δcya-12 Δcrp-11 derivatives χ3985 and χ4039.

Table 5

Virulence of S. typhimurium Δcya-12, Δcrp-11, Δcya-12, and Δcrp-11 Strains After Inoculation of BALB/c Mice with S. typhimurium Δcya-12 and/or Δcrp-11 Strains

Strain Number	Relevant Genotype	Route of Inoculation	Inoculating Dose (CFU)	Survival live/ Total	Health[a]	Approx. wild-type LD_{50}	Wild-type Origin
S. typhimurium							
χ3615	Δcya-12	PO	$2x10^9$	5/5	healthy	$6x10^4$	mouse
χ3623	Δcrp-11	PO	$5x10^8$	5/5	healthy	$6x10^4$	mouse
χ3985	Δcya-12 Δcrp-11	PO	$2x10^9$	8/10	moderate	$1x10^5$	horse
χ4039	Δcya-12 Δcrp-11	PO	$1x10^9$	10/10	healthy	$1x10^5$	pig
S. typhi							
χ3926	Δcya-12 Δcrp-11	IP[b]	$2x10^3$	4/6	healthy	~29	human
χ3927	Δcya-12 Δcrp-11	IP	$3x10^3$	2/4	healthy	<20	human

[a]Healthy-no noticeable signs of disease; moderate-moderately ill; ill-noticeably ill.

[b]IP-cells delivered in 0.5 ml 5% hog gastric mucin.

Effectiveness of immunization with avirulent mutants. Table 6 presents data on the ability of the S. typhimurium Δcya Δcrp mutants χ3985 and χ4039 to induce immunity to subsequent peroral challenge with 10^4 times the LD_{50} doses of fully virulent wild-type S. typhimurium cells. Under these high-dose challenges, many of the mice displayed moderate illness with decreased food consumption except mice immunized with χ4039 which remained healthy and ate and grew normally.

Table 6
Effectiveness of Immunization with Avirulent S. typhimurium Δcya-12 and/or Δcrp-11 Mutants in Protecting Against Challenge with Wild-type Virulent Parent Strains

Strain Number	Relevant Genotype	Dose (CFU) of Immunizing Strain	Dose (CFU) of Wild-type Challenge Strain	Survival live/ total
χ3615	Δcya-12	2×10^9	3×10^8	5/5
χ3623	Δcrp-11	5×10^8	3×10^8	5/5
χ3985	Δcya-12 Δcrp-11	2×10^9	7×10^8	8/8
χ4039	Δcya-12 Δcrp-11	1×10^9	6×10^8	10/10

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Example 3

This Example demonstrates the isolation of an avirulent microbe that possesses a deletion mutation encompassing the crp gene and an adjacent gene which also governs virulence of Salmonella.

Bacterial strains. The Escherichia coli and Salmonella typhimurium strains used are listed in Table 2A and B. The maintenance and storage of these strains are described in Example 1.

Media. Complex media for routine cultivation, enumeration and identification of bacteria are as described in Example 1.

Transduction and fusaric acid selection for loss of Tn10. The media and methods are as described in Example 1.

Animal infectivity and evaluation of protective immunity. The virulence and immunogenicity of S. typhimurium strains were determined as described in Example 1.

Isolation of S. typhimurium strain with the Δcrp-10 mutation. As described in Example 1, one of ten Δcrp mutations isolated in χ3605 conferred auxotrophy for arginine (due to deletion of argD) and cysteine (due to deletion of cysG). The mutation in the S. typhimurium SL1344 strain χ3622 was originally referred to as Δcrp-10 but is now designated Δ[crp-cysG]-10 because of the auxotrophy for cysteine. A group of five BALB/c mice orally infected with 10^9 χ3622 cells remained healthy and was totally unaffected (Table 4). Furthermore, these mice gained high-level immunity to oral challenge with 10^8 parental χ3339 cells (Table 4).

A series of strains was constructed to independently evaluate each of the phenotypic characteristics of χ3622. The plasmid, pSD110, carrying the cloned crp$^+$ gene and conferring ampicillin resistance (Schroeder and Dobrogosz, J. Bacteriol. 167:616-622 (1986)), was used to complement the Δcrp mutation in the chromosome. An L broth culture of

χ3622 was transduced with P22HTint propagated on S. typhimurium χ3670, which contains the plasmid pSD110. Selection was made on MacConkey agar + 1% maltose + 100 μg ampicillin/ml. After 26 h, an ampicillin-resistant, Mal⁺ colony was picked and purified on MacConkey agar + 1% maltose agar + 100 μg ampicillin/ml and designated χ3706. χ3706 was administered perorally to mice and reisolated from the spleen. The animal-passaged strain was designated χ3737. Two other crp mutants, χ3605 (crp-773::Tn10) and χ3623 (Δcrp-11) that do not confer the Arg⁻ or Cys⁻ auxotrophic traits were also complemented with the pSD110 plasmid by transduction and designated χ3731 and χ3774, respectively. S. typhimurium strains independently carrying cysG and arg mutations were constructed and designated χ3910 (cysG::Tn10), χ4063 and χ4071 (arg::Tn10).

Two other highly pathogenic S. typhimurium strains were selected for attenuation by introduction of the Δcrp-10 mutation. χ3761 (UK-1) and 798 are virulent, invasive strains isolated from a moribund horse and pig, respectively, with LD_{50}s in mice of approximately 1 x 10^5 CFU. Transduction of Δcrp-10 with the linked transposon zhc-1431::Tn10 was facilitated by first making a high-titer bacteriophage P22HTint lysate on the S. typhimurium strain χ3712 (see Table 2.B.). The phage lysate was then used to transduce the genetic traits into the wild-type recipient strains χ3761 and 798. Tetracycline-resistant colonies were selected and screened for the Mal⁺, Arg⁻ and Cys⁻ phenotypes and the resulting 798 derivative designated χ4246 and the χ3761 (UK-1) derivative designated χ4248 (Table 2).

The crp mutation was complemented by introducing pSD110, carrying the crp⁺ wild-type allele, into χ4246 and χ4248. L broth grown cultures of χ4246 and χ4248 were transduced with P22HTint propagated on S. typhimurium χ3670, which contains the plasmid pSD110 (Table 2). Selection was made on MacConkey agar + 1% maltose+ 100 μg

ampicillin/ml + 12.5 μg tetracycline/ml. After 26 h, an ampicillin, Mal⁺ colony of each strain was picked and purified on the same medium and designated χ4247 (798) and χ4262 (UK-1) which both have the genotype pSD110⁺/Δcrp-10 zhc-1431::Tn10.

Virulence of the S. typhimurium χ3622, χ3731, χ3737, χ3774, χ3910, χ4063 and χ4071. Table 7 presents data on morbidity and mortality of mice infected perorally with the S. typhimurium strains χ3622, χ3731, χ3737, χ3774, χ3910, χ4063 and χ4071. Strain χ3737 was completely avirulent for mice that received 10^4 times the LD_{50} dose for the wild-type χ3339 parent strain. Mice never appeared ill throughout the 30-day observation period. As a control for this experiment, the crp-773::Tn10 mutation in χ3605 was complemented by pSD110 to the wild-type Crp⁺ phenotype (χ3731) and mice were infected and died. Doses around 1 x 10^5 CFU killed 4 of 5 mice p.o. inoculated with χ3731 and χ3774 (pSD110⁺/Δcrp-11). To test the virulence of strains with the Cys⁻ and Arg⁻ phenotypes independently, strains χ3910 (cysG::Tn10), χ4063 (arg::Tn10) and χ4071 (arg::Tn10) were p.o. administered to BALB/c mice. χ3910, χ4063 and χ40671 killed mice when similar or lower doses were p.o. administered. Therefore, the avirulence associated with the Δ[crp-cysG]-10 mutation was not solely due to deletion of the crp gene and was not conferred by deletion of either the argD or cysG loci. Rather, another gene necessary for S. typhimurium virulence must be localized to the region of chromosome near the crp gene.

Table 7

Virulence of S. typhimurium SL1344 Δ(crp-cysG)-10, Crp$^+$/crp::Tn10 and Crp$^+$/Δ(crp-cysG)-10, arg::Tn10, cysG::Tn10 mutants in BALB/c mice 30 days after peroral inoculation

Strain number	Relevant genotype	Inoculating dose (CFU)	Survival live/total	Mean day of death[a]	Health[b]
χ3339	wild-type	6×10^4	2/5	7	scruffy
χ3622	Δ(crp-cysG)-10	6×10^8	5/5	-	healthy
χ3731	pSD110$^+$	1×10^5	1/5	9	scruffy
χ3737	crp-773::Tn10 pSD110+ Δ(crp-cysG)-10	5×10^8	5/5	-	healthy
χ3774	pSD110$^+$ Δcrp-11	3×10^4	3/5	12	scruffy
χ3910	cysG::Tn10	1×10^7	0/2	12	scruffy
χ4063	arg::Tn10	1×10^9	0/2	8	scruffy

Table 7 (cont'd.)

Virulence of S. typhimurium SL1344 Δ(crp-cysG)-10, Crp$^+$/crp::Tn10 and Crp$^+$/Δ(crp-cysG)-10, arg::Tn10, cysG::Tn10 mutants in BALB/c mice 30 days after peroral inoculation

Strain number	Relevant genotype	Inoculating dose (CFU)	Survival live/total	Mean day of death[a]	Health[b]
χ4071	arg::Tn10	1×10^9	0/2	9	scruffy

[a]of animals that died

[b]healthy-no noticeable signs of disease; moderate-moderately ill; scruffy-noticeably ill.

Effectiveness of immunization with χ3622, χ3737, χ4247 and χ4262. Data on the ability of χ3622, χ3737, χ4247 and χ4262 to induce immunity to subsequent p.o. or i.p. challenge with 10^4 times the LD_{50} doses of fully virulent wild-type S. typhimurium cells are presented in Table 7. All mice given excessive doses of the wild-type parent strain never appeared ill throughout the 30-day duration of the experiment. Therefore the Δ[crp-cyaC]-10 mutation deletes at least two genes both of which render S. typhimurium completely avirulent and highly immunogenic.

Table 8

Effectiveness of immunization with avirulent S. typhimurium Δ(crp-cyaC)-10 mutants in protecting against challenge with wild-type virulent parent strains

Strain number	Relevant genotype	Dose (CFU) of immunizing strain	Route of immunization	Dose (CFU) of wild-type strain	Survival live/total
χ3622	Δ[crp-cyaC]-10	6.2×10^8	PO	3.6×10^8	5/5
		1.5×10^9	PO	3.2×10^8	5/5
		4.2×10^8	PO	8.8×10^8	5/5
		9.0×10^6	IP	1.4×10^4	2/2
		9.0×10^4	IP	1.4×10^4	3/3
		9.0×10^2	IP	1.4×10^4	3/3
χ3737	pSD110⁺ Δ[crp-cyaC]-10	5.8×10^8	PO	8.4×10^8	5/5
χ3955	pSD110⁺ Δ[crp-cyaC]-11	6.8×10^8	PO	8.4×10^8	2/2

Table I (cont'd.)

Effectiveness of immunization with avirulent S. typhimurium Δ[crp-cysG]-10 mutants in protecting against challenge with wild-type virulent parent strains

Strain number	Relevant genotype	Dose (CFU) of immunizing strain	Route of immunization	Dose (CFU) of wild-type strain	Survival live/total
χ4247	pSD110⁺ Δ[crp-cysG]-10	2.0 x 10^9	PO	9.8 x 10^8	2/2
χ4262	pSD110⁺ [crp-cysG]-10	1.5 x 10^9	PO	5.4 x 10^8	3/3

Isolation of S. typhimurium strain with the Δcrp-14 mutation. Since an imprecise excision event of crp-773::Tn10 generated the deletion of genes extending from aroD through cysG, another strategy was designed to locate the position of the gene conferring avirulence in the region adjacent to crp. Twenty independent deletion mutants of χ3910 (cysG::Tn10) were selected on fusaric acid-containing medium and screened for tetracycline-sensitivity and maltose-negative phenotype. One of twenty fusaric acid-resistant derivative of χ3910 had the genotype Δ[crp-cysG]-14 and conferred auxotrophy for histidine and cysteine, but not arginine. This strain, designated χ3931, was transduced with a P22HTint lysate grown on χ3670 to introduce pSD110 carrying the wild-type crp⁺ gene. An ampicillin-resistant, maltose-positive transductant was picked and purified on the same medium and the resulting strain was designated χ3955.

Virulence of S. typhimurium pSD110⁺/Δ[crp-cysG]-14 χ3955. Table 8 shows morbidity and mortality of mice infected perorally with S. typhimurium χ3955. Strain χ3955 was completely avirulent for mice that received approximately 10^9 CFU. Mice never appeared ill throughout the 30-day period.

Effectiveness of immunization with χ3955. Table 8 shows the ability of χ3955 to induce immunity to subsequent p.o. challenge with 10^4 times the LD_{50} dose of fully virulent wild-type S. typhimurium cells. Mice given excessive doses of the parent strain never appeared ill throughout the 30-day duration of the experiment.

Colonization of intestinal tract, GALT and spleen by χ3622 (Δ[crp-cysG]-10) and χ3737 (pSD110⁺ Δ[crp-cysG]-10) relative to the wild-type strain χ3339. S. typhimurium χ3622 and χ3737 were grown and prepared for oral inoculation of 8-week-old female BALB/c mice as described in Example 1. Animals were sacrificed 1, 3, 5 and 7 days after p.o. inoculation with 9/4 x 10^8 CFU (χ3622), 1.2 x 10^9

CFU (χ3737) or 1.1 x 10^9 CFU (χ3339). Three mice per group were randomly selected, euthanized and tissue samples collected. The spleen, Peyer's patches, a 10-cm section of the ileum and the small intestinal contents from each mouse were placed in polypropylene tubes with BSG, homogenized with a Brinkmann tissue homogenizer and placed on ice. Undiluted or diluted samples (100 μl) were plated directly on MacConkey agar + 1% lactose + 50 μg streptomycin/ml (χ3339 and χ3737) and MacConkey agar + 1% maltose + 50 μg streptomycin/ml (χ3622) and the plates were incubated for 26 h 37°C. Titers in the perspective tissues were determined for each time period and the geometric mean calculated for 3 mice per group at each time of sampling.

The results of this analysis are presented in Figures 3 and 4. It is evident that the additional attenuating mutation in χ3622 and which is still manifested in the Crp$^+$ (pSD110$^+$) derivative χ3737 very much diminishes the ability to effectively colonize deep tissues. The responsible gene which is deleted by the Δ[crp-cysG]-10 mutation has therefore been designated cdt. The Cdt$^-$ phenotype of χ3622 and χ3737 is also manifested by the absence of any splenomegaly which is observed following p.o. inoculation of mice with S. typhimurium χ3623 which has the Δcrp-11 mutation or with various other strains with combined Δcrp and Δcya mutations (Curtiss and Kelly, 1987). Strain χ3737 grew more rapidly than χ3622. The additional attenuating mutation in χ3622 does not decrease growth rate as does the crp mutation.

Based on isolation and analysis of deletion mutations for phenotypes conferred, the order of genes in the S. typhimurium chromosome is inferred to be aroD crp cdt cysG.

It is evident that inclusion of the Δ[crp-cysG]-10 or Δ[crp-cysG]-14 mutations which are also Δcdt mutations would enhance the safety of live attenuated Salmonella vaccine strains while not diminishing their immunogenicity.

This might be particularly important for host-adapted invasive Salmonella species such as S. typhi, S. paratyphi, A (S. schottmuelleri), S. paratyphi B (S. hirshfeldii), S. paratyphi C (all infect humans), S. choleraesuis (infects swine), S dublin (infects cattle), S gallinarum, and S. pullorum (both infect poultry); as well as non-host specific, invasive Salmonella species such as S. typhimurium and S. enteritidis.

Example 4

This example describes the construction of avirulent microbes by the introduction of deletion mutations affecting cAMP synthesis and utilization and an adjacent gene which also governs virulence of Salmonella by affecting colonization of deep tissues and the characterization of strains with two deletion mutations for stability of phenotype, complete avirulence and high immunogenicity.

Bacterial strains. The Escherichia coli and Salmonella typhimurium strains used are listed in Table 2.A. and B. The maintenance and storage of these strains are as described in Example 1.

Media. Complex media for routine cultivation, enumeration and identification of bacteria are as described in Example 1.

Transduction and fusaric acid selection for loss of Tn10. The media and methods are as described in Example 1.

Construction of S. typhimurium strains with Δcya-12 and Δ[crp-cysG]-10 deletion mutations. The best vaccine strains in terms of efficacy are likely to result from the attenuation of highly virulent strains that display significant colonizing ability and invasiveness. The criteria for selection of these highly pathogenic S. typhimurium wild-type strains such as SL1344 (χ3339), UK-1 (χ3761) and 798 has been described in Example 2.

The wild-type, virulent S. typhimurium strains SL1344, 798 and UK-1 were genetically modified as described

below, using classical genetic methods similar to those described in Curtiss and Kelly (1987). The strategy consists of mobilizing deletions of crp and cya genes that have been isolated and characterized in S. typhimurium SL1344 (as described in Example 1) by placing the transposon Tn10 (encoding tetracycline resistance) nearby the Δcya-12 or Δ[crp-cysG]-10 mutation and transducing the linked traits into the highly virulent S. typhimurium strains UK-1 χ3761, 798 and SL1344 χ3339 via P22HTint-mediated transduction with selection for tetracycline resistance and screening for a maltose-negative phenotype. The zhc-1431::Tn10 linked to Δ[crp-cysG]-10 and zid-62::Tn10 linked to Δcya-12 were used for this purpose. Neither insertion alone affects the virulence of S. typhimurium.

Transduction of the gene deletions with the linked transposon was facilitated by first making a high-titer bacteriophage P22HTint lysate on the S. typhimurium strain χ3712 containing the Δ[crp-cysG]-10 and zhc-1431::Tn10 mutations and another lysate on the S. typhimurium strain χ3711 containing the Δcya-12 and zid-62::Tn10 mutations. The resulting P22HTint lysates were then used to transduce the genetic traits into the wild-type recipient strains χ3339, 798 and χ3761.

P22HTint propagated on S. typhimurium χ3712 (Δ[crp-cysG]-10 zhc-1431::Tn10) was used to transduce the virulent strains to tetracycline resistance with screening for Mal⁺. The phage-bacteria infection mixtures were incubated for 20 min at 37°C before 100 μl samples were spread onto MacConkey agar (Difco Laboratories, Detroit, MI) containing 1% maltose (final concentration) supplemented with 12.5 μg tetracycline/ml. After approximately 26 h incubation at 37°C, tetracycline resistant Mal⁺ transductants were picked and purified onto the same medium. The resulting 798 derivative was designated χ3777 and the UK-1 derivative was designated χ3779. Strains χ3712, χ3777 and χ3779 all have

the genotype Δ[crp-cysG]-10 zhc-1431::Tn10 (Table 2.B.). χ3777 and χ3779 were grown in L broth + 12/5 μg tetracycline/ml and each were diluted 1:10 into buffered saline with gelatin (BSG), 100 μl of each were spread onto fusaric acid-containing (FA) media (Maloy and Nunn, 1981) and the plates were incubated approximately 36 h at 37°C. Fusaric acid-resistant colonies of each strain were picked into 0.5 ml BSG and purified onto FA medium. Purified fusaric acid-resistant colonies were picked into L broth and grown at 37°C to turbidity and checked for loss of Tn10 (tetracycline sensitivity), presence of complete LPS and auxotrophy. The new strains were designated χ3784 (UK-1) and χ3806 (798) which both have the genotype Δ[crp-cysG]-10 Δ[zhc-1431::Tn10]. χ3622 (SL1344) Δ[crp-cysG]-10) was originally isolated as described in Example 1) (Table 2B).

Since the phenotype of Cya⁻ and Crp⁻ mutants are the same (Mal⁻, Stl⁻, Mtl⁻, etc.), the plasmid, pSD110, carrying the cloned crp⁺ gene and conferring ampicillin resistance (Schroeder and Dobrogosz, J. Bacteriol 167:616-622 (1986)), was used to temporarily complement the Δcrp mutation in the chromosome enabling the identification of the Δcya mutation when introduced via transduction. L broth grown cultures of χ3622, χ3784 and χ3806 were transduced with P22HTint propagated on S. typhimurium χ3670, which contains the plasmid pSD110 (Table 2). Selection was made on MacConkey agar + 1% maltose + 100 μg ampicillin/ml. After 26 h, an ampicillin-resistant, Mal⁺ colony of each strain was picked and purified on MacConkey agar + 1% maltose agar + 100 μg ampicillin/ml and designated χ 3901 (798) and χ3945 (UK-1) which both have the genotype Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] pSD110⁺ and χ3706 (SL1344) which has the genotype Δ[crp-cysG]-10 pSD110⁺.

Strains χ3706, χ3901 and χ3945 were grown in L broth + 100 μg ampicillin/ml and were each independently transduced with P22HTint propagated on χ3711 to introduce the linked Δcya-12 and zid-62::Tn10 mutations. The

Media. Complex media for routine cultivation, enumeration and identification of bacteria are as described in Example 1.

Transduction and fusaric acid selection for loss of Tn10. The media and methods are as described in Example 1.

Genetic stability of avirulent mutants. Methods for determining stability of genetic traits are as described in Example 1.

Mice. Female CFW-1 mice (18-20 g) (Charles River, Wilmington, MA) were used for all infectivity experiments. Animals were held for one week in a quarantined room prior to being used in experiments. Experimental mice were placed in Nalgene filter-covered cages with wire floors. Food and water were given ad libitum. The animal room was maintained at 22-23°C with a period of 12 h illumination.

Animal infectivity. The virulence of S. typhi strains was determined following intraperitoneal (i.p.) injection with hog gastric mucin. Bacteria for inoculation into mice were grown overnight as standing cultures at 37°C in L broth. The cultures were diluted 1:50 into prewarmed L broth and aerated at 37°C for approximately 4 h to an OD_{600} of about 0.8-1.0. Suitable dilutions were plated on Penassay agar for titer determination and on MacConkey agar with 1% maltose to verify the Cya/Crp phenotype.

Intraperitoneal inoculation of unfasted CFW-1 mice was performed using a 26-gauge 3/8" needle to deliver 500 µl of S. typhi cells suspended in 15% (w/v) hog gastric mucin (Wilson lot #0347A001). The mucin suspension was prepared by autoclaving 10 min 121°F (15 p.s.i.), neutralizing to pH 7 and adding 3 µg of ferric ammonium citrate (Sigma, St. Louis, MO) per ml prior to adding S. typhi cells. LD_{50} values of the wild-type parents and virulence of the Δcrp-11 Δcya-12 derivatives were determined after recording morbidity and mortality data for 10 days.

Construction of S. typhi strains with cya and crp mutations. The wild-type, virulent S. typhi Ty2 (type E1), ISP1820 (type 46) and ISP2822 (type E1) strains were genetically modified as described below, using classical genetic methods similar to those described in Curtiss and Kelly (1987). ISP1820 and ISP2822 were recently isolated during a typhoid epidemic in Chile and are likely to be more invasive than the standard laboratory Ty2 strain of S. typhi. Their attenuation might therefore generate vaccine strains that would be more efficacious than those derived from Ty2. The construction strategy consists of mobilizing deletions of crp and cya genes that have been isolated and characterized in S. typhimurium SL1344 by placing the transposon Tn10 (encoding tetracycline resistance) nearby the Δcya or Δcrp mutation and transducing the linked traits into the highly virulent S. typhi Ty2, ISP1820 and ISP2822 strains via P22HTint-mediated transduction with selection for tetracycline resistance and screening for a maltose-negative phenotype. The zhc-1431::Tn10 linked to crp and zid-62::Tn10 linked to cya were used for this purpose. Neither insertion alone affects virulence of S. typhimurium.

Transduction of the gene deletions with the linked transposon was facilitated by first making a high-titer bacteriophage P22HTint lysate on the S. typhimurium strain χ3773 containing the Δcrp-11 and zhc-1431::Tn10 mutations and another lysate on the S. typhimurium strain χ3711 containing the Δcya-12 and zid-62::Tn10 mutations. The resulting P22HTint lysates were then used to infect at a multiplicity of infection of 10 to transduce the genetic traits into the recipient S. typhi Ty2, ISP1820 and ISP2822 strains.

P22HTint propagated on S. typhimurium χ3773 (Δcrp-11 zhc-1431::Tn10) was used to transduce the virulent S. typhi Ty2, ISP1820 and ISP2822 strains to tetracycline resistance with screening for Mal⁻. The phage-bacteria infection

mixtures were incubated for 20 min at 37°C before 100 µl samples were spread onto MacConkey agar (Difco Laboratories, Detroit, MI) containing 1% maltose (final concentration) supplemented with 12.5 µg tetracycline/ml. After approximately 26 h incubation at 37°C, tetracycline-resistant Mal⁻ transductants were picked and purified onto the same medium. The resulting Ty2 derivative was designated χ3853, the ISP1820 derivative designated χ3298 and the ISP2822 derivative designated χ3852. All of these strains have the genotype Δcrp-11 zhc-1431::Tn10 (Table 2.C.). Strains χ3852, χ3853 and χ4298 were grown in L broth + 12.5 µg tetracycline/ml and each were diluted 1:10 into buffered saline with gelatin (BSG), 100 µl of each were spread onto fusaric acid-containing (FA) media (Maloy and Nunn, 1981) and the plates were incubated approximately 36 h at 37°C. Fusaric acid-resistant colonies of each strain were picked into 0.5 ml BSG and purified onto FA medium. Purified fusaric acid-resistant colonies were picked into L broth and grown at 37°C to turbidity and checked for loss of Tn10 (tetracycline sensitivity), presence of complete LPS and Vi antigen and auxotrophy for cysteine and tryptophan (two amino acids required by all the parent strains). The new strains were designated (ISP2822), χ3878 (Ty2) and χ4299 (ISP1820) which all have the genotype Δcrp-11 Δ[zhc-1431::Tn10] (Table 2.C.).

Since the phenotype of Cya⁻ and Crp⁻ mutants are the same (Mal⁻, Stl⁻, Mtl⁻, etc.), the plasmid, pSD110, carrying the cloned crp⁺ gene conferring ampicillin resistance (Schroeder and Dobrogosz, J. Bacteriol. 167:616-622 (1986)), was used to temporarily complement the Δcrp mutation in the chromosome enabling the identification of the Δcya mutation when introduced via transduction. L broth grown cultures of χ3877, χ3878 and χ4299 were transduced with P22HTint propagated on S. typhimurium χ3670, which contains the plasmid pSD110 (Table 2.B.). Selection was made on MacConkey agar + 1%maltose + 100 µg

ampicillin/ml. After 26 h, an ampicillin-resistant, Mal⁺ colony of each strain was picked and purified on MacConkey agar + 1% maltose agar + 100 µg ampicillin/ml and designated χ3879 (ISP2822), χ3880 (Ty2) and χ4300 (ISP1820) which all have the genotype Δcrp-11 Δ[zhc-1431::Tn10] pSD110⁺.

Strains χ3879, χ3880 and χ4300 were grown in L broth + 100 µg ampicillin/ml and were each independently transduced with P22HTint propagated on χ3711 to introduce the linked Δcya-12 and zid-62::Tn10 mutations. The transduction mixtures were plated on MacConkey agar + 1% maltose + 100 µg ampicillin/ml + 12.5 µg tetracycline/ml. Ampicillin-resistant (pSD110⁺), tetracycline-resistant (zid-62::Tn10), Mal⁻ (Δcya) colonies were picked and purified on MacConkey agar + 1% maltose + 100 µg ampicillin/ml + 12.5 µg tetracycline/ml. Purified colonies were picked into L broth, grown to turbidity and the strains checked for complete LPS, Vi antigen and auxotrophy for cysteine and tryptophan. The resulting strains were designated χ3921 (ISP2822), χ3922 (Ty2) and χ4316 (ISP1820) which all have the genotype Δcrp-11 Δ[zhc-1431::Tn10] pSD110⁺ Δcya-12 zid-62::Tn10 (Table 2.C.). Cultures of χ3921, χ3922 and χ4316 were grown in L broth + 100 µg ampicillin/ml + 12.5 µg tetracycline/ml to turbidity, diluted 1:10 into BSG, and 100 ml samples of each culture spread onto fusaric-containing media and incubated approximately 36 h at 37°C. Fusaric acid-resistant colonies of each strain were picked and purified onto FA medium. Purified FA-resistant colonies were picked into L broth, grown to turbidity and then checked for loss of Tn10 (tetracycline sensitivity), complete LPS, Vi antigen and auxotrophy for cysteine and tryptophan. The pSD110 plasmid was usually spontaneously lost from the strains during this process to result in ampicillin sensitivity. The final strains were designated χ3926 (ISP2822), χ3927 (Ty2) and χ4322 (ISP1820) which all have the genotype Δcrp-11 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-

62::Tn10 (Table 2.C.). S. typhi Vi antigen synthesis was confirmed by slide agglutination with antisera to Vi (Difco Laboratories, Detroit, MI) and by ViII bacteriophage sensitivity by the Luria soft agar overlay technique. Synthesis of flagella is dependent on functional cya and crp genes. However, since flagella are a potentially important antigen, motile derivatives of Δcya Δcrp S. typhi strains, due to mutation in the cfs (constitutive flagellar syntheses) gene (Silverman and Simon, J. Bacteriol. 120:1196-1203 (1974)), were selected in motility agar. χ3926 and χ3927 were isolated as flagellated and motile whereas strain χ4323 was selected as a flagella-positive motile derivative of χ4222.

Table 9 lists the phenotypic properties of all the mutant strains and their parents with regard to fermentation of sugars and growth on various carbon sources, LPS profile, Vi antigen and mean generation time. The phenotypes are as expected based on published reports of the requirement for cyclic AMP and the cyclic AMP receptor protein for catabolic activities.

TABLE 9. Fermentation and growth properties of S. typhi strains

		Phenotype			
		χ1745	χ3926	χ3762	χ3927
MacConkey Base Agar	+ 1% maltose	+	-	+	-
"	+ 1% sorbitol	+	-	+	-
"	+ 1% mannitol	+	-	+	-
"	+ 1% melibiose	+	-	+	-
"	+ 1% rhamnose	-	-	-	-
"	+ 1% citrate	-	-	-	-
"	+ 1% arabinose	-	-	-	-
"	+ 1% mannose	+	+	+	-
"	+ 1% xylose	+	-	+	-
"	+ 1% glucose	+	+	+	+
Minimal agar	+ 0.5% glucose	+	+	+	+
"	+ 0.5% sorbitol	+	-	+	-
"	+ 0.5% mannitol	+	-	+	-
"	+ 0.5% melibiose	+	-	+	-
"	+ 0.5% rhamnose	-	-	-	-
"	+ 0.5% citrate	-	-	-	-
"	+ 0.5% arabinose	-	-	-	-

TABLE 9. Fermentation and growth properties of S. typhi strains (continued)

	Phenotype			
	x1745	x1926	x1769	x1927
Motility[bc]	+	+	+	+
Colicin(s) production	-	-	-	-
MGT[c]		21.5	26.2	24.337.1
Plasmid content	none	none	none	none
Auxotrophy	Cys^-	Cys^-	Cys^-	Cys^-
	Trp^-	Trp^-	Trp^-	Trp^-
MIC[d]				
Tetracycline	4	4	<2	4
Streptomycin	64	64	16	8

[a]phage sensitivity was assayed by soft agar overlay technique or by transduction. S - sensitive; R = resistant.

[b]Motility determined by stabbing a loopful of a standing overnight culture into media containing 1.0% casein, 0.5% $NaCl_2$, 0.5% Difco agar, 50 µg/mg triphenyltetrazolium chloride indicator agar; incubation at 37°C and motility recorded at 24 and 48 h.

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TABLE 9. Fermentation and growth properties of S. typhi strains (continued)

	Phenotype			
	x1745	x1926	x1769	x1927
Minimal agar (continued)	+ 0.5% mannose		+	++-
"	+ 0.5% xylose		+	-+-
Triple Sugar Iron media - H_2S production	+	-	+	-
alkaline slant /	Lac^-	Lac^-	Lac^-	Lac^-
	Glu^+	Glu^+	Glu^+	Glu^+
	Suc^-	Suc^-	Suc^-	Suc^-
Indole fermentation assay	-	-	-	-
Bacteriophage sensitivity[a]				
ViII S	S	S	S	
Felix-0 S	S	S	S	
P22HTint S	S	S	S	
P1L4 R	R	R	R	
L R	R	R	R	
KB1 R	R	R	R	
LPS profile by SDS-PAGE (silver strain)	complete	complete	complete	complete

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TABLE 9. Fermentation and growth properties of S. typhi strains (continued)

[c]Mean Generation Time (min) = determined in Luria broth with aeration (150 rpm New Brunswick platform shaker) at 37°C.

[d]Minimal inhibitory concentrations (μg/ml) of antibiotics were determined by streaking standing overnight cultures of each strain onto agar containing defined concentrations of antibiotics.

Genetic stability of avirulent mutants. Strains to be orally administered as live vaccines must have complete stability with regard to their avirulence attributes. When 50-fold concentrated cultures and various dilutions (~10^9, 10^7, 10^5, 10^3 CFU/plate) of the Δcya Δcrp S. typhi strains were plated on minimal agar media (supplemented with required amino acids) containing 0.5% maltose, melibiose, xylose, glycerol, or rhamnose that should not support their growth, revertants and mutants were not detected. One set of duplicate plates was UV-irradiated (5 joules/meter^2/sec) and incubated at 37°C in the dark. The other set of plates was incubated at 37°C with illumination. Revertants and mutants were not detected after a 48 h growth period. An investigation was also conducted as to whether tetracycline-resistant revertants/mutants could be recovered at frequencies higher than could be observed for the parental strain. In all cases, such tetracycline-resistant revertants/mutants were not observed.

Virulence of mutant strains for mice. Mice survive infection with about 10^4 times the LD_{50} dose of either χ3926 or χ3927. The natural host for S. typhi is man. Therefore, hog gastric mucin is used as a virulence enhancer of S. typhi cells in mice, and thus maximizes the virulence of S. typhi vaccine candidates in this model system.

Example 6

This example demonstrates the construction of avirulent microbe by the introduction of deletion mutations affecting cAMP synthesis and utilization and an adjacent gene which governs virulence of Salmonella by affecting colonization of deep tissues.

Bacterial strains. The Salmonella typhimurium and S. typhi strains used are listed in Table 2.B. and C. The maintenance and storage of these strains are as described in Example 1.

Media. Complex media for routine cultivation, enumeration and identification of bacteria are as described in Example 1.

Transduction and fusaric acid selection for loss of Tn10. The media and methods are as described in Example 1.

Genetic stability of avirulent mutants. Methods for determining stability of genetic traits are as described in Example 1.

Construction of S. typhi strains with Δcya-12 and Δ[crp-cysG]-10 mutations. S. typhi is highly invasive for humans. Although S. typhi strains with the Δcya-12 and Δcrp-11 mutations appear to be avirulent, it would seem prudent to consider adding an additional attenuating mutation to further enhance safety without compromising immunogenicity. The properties of the Δ[crp-cysG]-10 mutation in S. typhimurium strains (Examples 1, 3, and 4) justify its use to render S. typhi avirulent and immunogenic. This mutation also deletes the cdt gene governing colonization of deep tissues by Salmonella typhimurium without significantly diminishing colonization of the intestinal tract and GALT.

The wild-type, virulent Ty2 (type E1), ISP1820 (type 46) and ISP2822 (type E1) strains were genetically modified as described below, using classical genetic methods similar to those described in Curtiss and Kelly (1987). ISP1820 and ISP2822 were recently isolated during a typhoid

epidemic in Chile and are likely to be more invasive than the standard laboratory Ty2 strain of S. typhi. Their attenuation might therefore generate vaccine strains that could be more efficacious than those derived from Ty2. The construction strategy consists of mobilizing deletions of crp and cya genes that have been isolated and characterized in S. typhimurium SL1344 (as described in Example 1) by placing the transposon Tn10 (encoding tetracycline resistance) nearby the Δcya or Δ[crp-cysG]-10 mutation and transducing the linked traits into S. typhi Ty2 and the highly virulent S. typhi ISP1820 and ISP2822 strains via P22HTint-mediated transduction with selection for tetracycline resistance and screening for a maltose-negative phenotype. This zhc-1431::Tn10 linked to [crp-cysG]-10 and zid-62::Tn10 linked to cya were used for this purpose. Neither insertion alone affects virulence of S. typhimurium.

Transduction of the gene deletions with the linked transposon was facilitated by first making a high-titer bacteriophage P22HTint lysate on the S. typhimurium strain χ3712 containing the Δ[crp-cysG]-10 and zhc-1431::Tn10 mutations and another lysate on the S. typhimurium strain χ3711 containing the Δcya-12 and zid-62::Tn10 mutations. The resulting P22HTint lysates when then used to transduce the genetic traits into the recipient S. typhi Ty2, ISP1820 and ISP2822 strains.

P22HTint propagated on S. typhimurium χ3712 (Δ[crp-cysG]-10 zhc-1431::Tn10) was used to transduce the virulent S. typhi Ty2, ISP1820 and ISP2822 strains to tetracycline resistance with screening for Mal⁻. The phage-bacteria infection mixtures were incubated for 20 min at 37°C before 100 μl samples were spread onto MacConkey agar (Difco Laboratories, Detroit, MI) containing 1% maltose (final concentration) supplemented with 12.5 μg tetracycline/ml. After approximately 26 h incubation at 37°C, tetracycline-resistant Mal⁻ transductants were picked and purified onto

the same medium. The resulting ISP2822 derivative was designated χ3791, the Ty2 derivative was designated χ3792, and the ISP1820 derivative was designated χ4324. All of these strains have the genotype Δ[crp-cysG]-10 zhc-1431::Tn10 and were auxotrophic for cysteine, tryptophan and arginine (Table 2.C.). Strains χ3791, χ3792 and χ4324 were grown in L broth + 12.5 μg tetracycline/ml. Each culture was diluted 1:10 into buffered saline with gelatin (BSG), 100 μl of each was spread onto fusaric acid-containing (FA) media (Maloy and Nunn, 1981) and the plates incubated approximately 36 h at 37°C. Fusaric acid-resistant colonies of each strain were picked into 0.5 ml BSG and purified onto FA medium. Purified fusaric acid-resistant colonies were picked into L broth and grown at 37°C to turbidity and checked for loss of Tn10 (tetracycline sensitivity), presence of complete LPS and Vi antigen and auxotrophy for cysteine, arginine and tryptophan. The new strains were designated χ3802 (ISP2822), χ3803 (Ty2) and χ4325 (ISP1820) which all have the genotype Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] (Table .C.).

Since the phenotype of Cya⁻ and Crp⁻/Cdt⁻ mutants are the same (Mal⁻, Stl⁻, Mtl⁻, etc.), the plasmid, pSD110, carrying the cloned crp⁺ gene and conferring ampicillin resistance (Schroeder and Dobrogosz, J. Bacteriol. 167:616-622 (1986)), was used to temporarily complement the Δcrp mutation in the chromosome enabling the identification of the Δcya mutation when introduced via transduction. L broth grown cultures of χ3802, χ3803 and χ4325 were transduced with P22HTint propagated on S. typhimurium χ3670, which contains the plasmid pSD110 (Table 2.B.). Selection was made on MacConkey agar + 1% maltose + 100 μg ampicillin/ml. After 26 h, an ampicillin-resistant, Mal⁺ colony of each strain was picked and purified on MacConkey agar. + 1% maltose agar + 100 μg ampicillin/ml and designated χ3824 (Ty2), χ3945 (ISP2822) and χ4331 (ISP1820)

which all have the genotype Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] pSD110⁺.

Strains χ3824, χ3845, and χ4331 were grown in L broth + 100 μg ampicillin/ml and were each independently transduced with P22HTint propagated on χ3711 to introduce the linked Δcya-12 and zid-62::Tn10 mutations. The transduction mixtures were plated on MacConkey agar + 1% maltose + 100 μg ampicillin/ml + 12.5 μg tetracycline/ml. Ampicillin-resistant (pSD110⁺), tetracycline-resistant (zid-62::Tn10), Mal⁻ (Δcya) colonies were picked and purified on MacConkey agar + 1% maltose + 100 μg ampicillin/ml + 12.5 μg tetracycline/ml. Purified colonies were picked into L broth, grown to turbidity and the strains checked for complete LPS, Vi antigen and auxotrophy for cysteine and tryptophan. The resulting strains were designated χ3919 (Ty2), χ3920 (ISP2822) and χ4340 (ISP1820) which all have the genotype Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] pSD110⁺ Δcya-12 zid-62::Tn10. Cultures of χ3919, χ3920 and χ4340 were grown in L broth + 100 μg ampicillin/ml + 12.5 μg tetracycline/ml to turbidity, diluted 1:10 into BSG, and 100 μl samples of each culture spread onto fusaric acid-containing media and incubated approximately 36 h at 37°C. Fusaric acid-resistant colonies of each strain were picked and purified onto FA medium. Purified FA-resistant colonies were picked into L broth, grown to turbidity and then checked for loss of Tn10 (tetracycline sensitivity), complete LPS, Vi antigen and auxotrophy for cysteine, arginine and tryptophan. The pSD110 plasmid was usually spontaneously lost from the strains during this process to result in ampicillin sensitivity. The final strains were designated χ3924 (Ty2), χ3925 (ISP2822) and χISP1820) which all have the genotype Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10] (Table 2.C.). S. typhi Vi antigen synthesis was confirmed by slide agglutination with antisera to Vi (Difco Laboratories, Detroit, MI) and by ViII bacteriophage sensitivity by the Luria soft agar

overlay technique. Synthesis of flagella is dependent on functional cya and crp genes. However, since flagella are a potentially important antigen, motile derivatives of Δcya Δcrp S. typhi strains, due to mutation in the cfs (constitutive flagellar synthesis) gene (Silverman and Simon, J. Bacteriol. 120:1196-1203 (1974), were selected in motility agar. Strains χ3940 (ISP2822), χ4073 (Ty2) and χ4346 (ISP1820) were selected as flagella-positive motile derivatives of χ3925, χ3924 and χ4345, respectively.

Fermentation of sugars and growth on various carbon sources of the Δ[crp-cysG]-10 mutant strains were the same as observed for the Δcrp-11 mutant strains. The phenotypes are as expected based on published reports of the requirement for cyclic AMP and the cyclic AMP receptor protein for catabolic activities.

Genetic stability of avirulent mutants. Strains to be orally administered as live vaccines must have complete stability with regard to their avirulence attributes. When 50-fold concentrated cultures and various dilutions (10^9, 10^7, 10^5, 10^3 CFU/plate) of the Δcya Δcrp S. typhi strains were plated on minimal agar media (supplemented with required amino acids) containing 0.5% maltose, melibiose, xylose, glycerol, or rhamnose that should not support their growth, revertants and mutants were not detected. One set of duplicate plates was UV-irradiated (5 joules/meter^2/sec) and incubated at 37°C in the dark. The other set of plates was incubated at 37°C with illumination. Revertants and mutants were not detected after a 48 h growth period. An investigation was also conducted as to whether tetracycline-resistant revertants/mutants could be recovered at frequencies higher than could be observed for the parental strain. In all cases, such tetracycline-resistant revertants/mutants were not observed.

Example 7

This Example describes the construction of recombinant avirulent S. typhi strains expressing foreign

antigens for use as oral vaccines to immunize against various infectious diseases.

Bacterial strains. The E. coli, S. typhimurium and S. typhi strains used are listed in Table 2. The maintenance and storage of these strains are as described in Example 1.

Media. Complex media for routine cultivation, enumeration and identification of bacteria are as described in Example 1.

Transduction and fusaric acid selection for loss of Tn10. The media and methods are as described in Example 1.

Construction of S. typhi strains with AasdAl mutation. The wild-type, virulent S. typhi Ty2 (type E1) was genetically modified as described below, using classical genetic methods similar to those described in Curtiss and Kelly (1987) and Nakayama, Kelly and Curtiss (1988). The construction of strains χ3927 and χ4323 containing the Δcya-12 Δcrp-11 mutations was described in Example 5. The construction of strain χ4346 containing the Δ[crp-cysG]-10 mutations was described in Example 6. The stable maintenance and high-level expression of cloned genes on recombinant plasmids in avirulent Salmonella strains is dependent upon use of a balanced-lethal host-vector system. For this, a chromosomal mutation of the asd gene encoding aspartate β-semialdehyde dehydrogenase is introduced into a Δcya Δcrp mutant to impose an obligate requirement for diaminopimelic acid (DAP) which is an essential constituent to the rigid layer of the bacterial cell wall and which is not synthesized in animals. The chromosomal Δasd mutation is then complemented by a plasmid cloning vector possessing the wild-type asd$^+$ gene. Loss of the plasmid results in DAPless death and cell lysis. Such balanced-lethal host-vector combinations are stable for several weeks in the immunized animal host and elicit strong immune responses against the cloned gene product as well as against Salmonella.

The construction strategy consists of mobilizing the ΔasdA1 mutation that has been isolated and characterized in S. typhimurium LT2-z (χ3520) into a Δcya Δcrp S. typhi strain. This was accomplished by placing the transposon Tn10 (encoding tetracycline resistance) nearby the ΔasdA1 mutation and transducing the linked traits into the S. typhi Ty2 Δcya-12 Δcrp-11 strain χ3927, the S. typhi ISP1820 Δcya-12 Δcrp-11 strain χ4323 and the S. typhi ISP1820 Δcya-12 Δ[crp-cysG]-10 strain χ4346 via P22HTint transduction with selection for tetracycline resistance and screening for a diaminopimelic acid (DAP) -negative phenotype. The zhf-4::Tn10 linked to ΔasdA1 was used for this purpose.

Transduction of the gene deletion with the linked transposon was facilitated by first making a high-titer bacteriophage P22HTint lysate on S. typhimurium χ3520 containing the ΔasdA1 and zhf-4::Tn10 mutations. The resulting P22HTint lysate was then used to infect and transduce the genetic traits into the recipient S. typhi Ty2 strain χ3927, the ISP1820 strains χ4323 and χ4346 at a multiplicity of infection of 10.

The phage-bacteria infection mixture was incubated for 20 min at 37°C before 100 μl samples were spread onto Penassay agar (Difco Laboratories, Detroit, MI) containing 50 μg DAP/ml and supplemented with 12.5 μg tetracycline/ml. After approximately 26 h incubation at 37°C, transductants were picked and purified on the same medium. A screening of five tetracycline-resistant colonies yields approximately four to five transductants that are also DAP-requiring. The resulting Ty2 derivative was designated χ4296 and has the genotype Δcrp-11 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10] ΔasdA1 zhf-4::Tn10. The resulting ISP1820 derivatives were designated χ4416 with the genotype Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] Δzid-62::Tn10] ΔasdA1 zhf-4::Tn10 and χ4434 with the genotype Δcrp-11 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10] ΔasdA1 zhf-4::Tn10.

Strains χ4296, χ4416 and χ4434 were grown in L broth + 50 μg DAP/ml + 12.5 μg tetracycline/ml and was diluted 1:10 into buffered saline with gelatin (BSG), 100 μl was spread onto fusaric acid-containing (FA) + 50 μg DAP/ml medium (Maloy and Nunn, 1981) and the plates were incubated approximately 36 h at 37°c. Fusaric acid-resistant colonies were picked into 05 ml BSG and purified onto FA + 50 μg DAP/ml media. Purified fusaric acid-resistant colonies were picked into L broth + 50 μg DAP/ml and grown at 37°C to turbidity and checked for loss of Tn10 (tetracycline sensitivity), presence of complete LPS and Vi antigen and auxotrophy for cysteine, tryptophan, methionine, threonine and DAP on minimal media. The new strains were designated χ4297 (Ty2), which has the genotype Δcrp-11 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10] ΔasdA1 Δ[zhf-4::Tn10]; χ4417 (ISP1820), which has the genotype Δ[crp-cysG]-10 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10] ΔasdA1 Δ[zhf-4::Tn10]; and χ4435 (ISP1820), which has the genotype Δcrp-11 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10] ΔasdA1 Δ[zhf-4::Tn10].

Asd⁻ derivatives of the wild-type parent strains were constructed for the purpose of compering the production of a recombinant antigen expressed by a Crp⁺ Cya⁺ background versus a Crp⁻ Cdt⁻ Cya⁺ background. The Ty2 ΔasdA1 strain was constructed by cotransducing S. typhi Ty2 strain X3769 and the S. typhi ISP1820 strain Δ3744 with P22HTint (X3520), selecting tetracycline resistance and screening for a diaminopimelic acid-negative phenotype. The resulting Ty2 derivative was designated X4456 and the ISP1820 derivative was designated X4454 and both have the genotype ΔasdA1 zhf-4::Tn10. Strains X4456 and X4454 were grown in L broth + 50 μg DAP/ml + 12.5 μg tetracycline/ml and was diluted 1:10 into buffered saline with gelatin (BSG), a 100 μl sample was spread onto fusaric acid containing + 50 μg DAP/ml medium (Maloy and Nunn, 1981), and the plates were incubated approximately 35 h at 37°C.

Fusaric acid-resistant colonies were picked into L broth + 50 μg DAP/ml and grown at 37°C to turbidity and checked for loss of Tn10 (tetracycline sensitivity), presence of complete LPS and Vi antigen, and auxotrophy for cysteine, tryptophan, methionine, threonine and DAP on minimal media. The new strains were designated X4457 (Ty2) and X4455 and have the genotype ΔasdA1 Δ(zhf-4::Tn10).

Expression of a Mycobacterium leprae antigen in avirulent recombinant S. typhi. λgt11::Mycobacterium leprae clone L14 (also designated clone 7.8) was identified by immunological screening of a λgt11::M. leprae library with pooled sera from 21 lepromatous (LL) leprosy patients (Sathish, Esser, Thole and Clark-Curtiss, Infect. Immun. 58: 1327-1336 (1990)). Clone L14 specifies two proteins of approximately 158 and 153 kDa, both of which react very strongly with antibodies in the pooled LL patients' sera (Sathish et al., 1990). These proteins also react with antibodies in 14 out of the 21 LL patients' sera when the sera were tested individually (Clark-Curtiss, Thole, Sathish, Bosecker, Sela, de Carvalho and Esser, Res. in Microbiology, in press).

The 1.0 kb M. leprae insert DNA fragment was removed from λgt11 clone L14 by digestion of the recombinant phage DNA with EcoRI, followed by separation of the digestion fragments by agarose gel electrophoresis. The M. leprae fragment was purified from the gel and cloned into the EcoRI site of the Asd⁺ vector pYA292 (Galan, Nakayama and Curtiss, Gene (1990), 94:29). Two kinds of recombinant plasmids were generated: pYA1077, in which the M. leprae insert DNA was cloned into pYA292 in the same orientation relative to the trc promoter as it was in λgt11 relative to the lacZ promoter, and pYA1078, in which the M. leprae fragment was cloned in the opposite orientation relative to the trc promoter. A partial restriction map of pYA1077 is presented in Figure 5. Both recombinant plasmids were transformed into Escherichia coli K-12 strain χ6060 and S.

typhimurium strain χ3730 and the proteins specified by the transformants were analyzed by Western blotting. Clone pYA1077 specifies a single fusion protein of approximately 30 kDa, which reacts strongly with antibodies in the pooled LL patients' sera. Clone pYA1078 does not specify any protein that reacts with the patients' sera.

Bacteriophage P22HTint lysates were prepared on S. typhimurium χ3730 + pYA1077 and χ3730 + pYA1078; these lysates were used to transduce S. typhi χ4297, χ4417, χ4435, χ4455, and Ψ4457. Western blot analysis of the proteins produced by three randomly chosen transductants of χ4297 with pYA1077 showed that each transductant specified a protein of 30 kDa that reacted with the pooled LL patients' sera whereas three independent χ4297 transductants harboring pYA1078 did not specify an immunologically reactive protein (Figure 6).

In addition, expression of immunologically reactive proteins from pYA1077 was also shown in χ4417, χ4435, χ4455, and χ4457. Figure 7 shows a Western blot of proteins produced by λgt11:M. leprae clone L14 and S. typhi, S. typhimurium and E. coli strains harboring pYA292, pYA1077 and pYA1078. The proteins on the nitrocellulose filter were reacted with pooled sera from 21 lepromatous leprosy patients. Positive antigen-antibody were detected by the technique described by Sathish, Esser, Thole and Clark-Curtiss (1990) 58::1327. More specifically, the secondary antibody was alkaline phosphatase-conjugated anti-human polyspecific antibodies and the chromogenic substrates were nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt. The lanes in the figures are as follows: (lane 1) molecular size markers; (lane 2) S. typhi χ4297 with pYA1077; (lane 3) S. typhi χ4417 with pYA1077; (lane 4), S. typhi χ4435 with pYA1077; (lane 5) S. typhi Ψ4455 with pYA1077; (lane 6) S. typhi χ4457 with pYA1077; (lane 7) S. typhi χ4297 with pYA292; (lane 8) S. typhi χ4435 with pYA292; (lane 9) S.

typhi χ4455 with pYA292; (lane 10) S. typhi χ4457 with pYA292; (lane 11) S. typhi χ4417 with pYA292; (lane 12) E. coli χ6097 with pYA1077; (lane 13) proteins from λgt11::M. leprae clone L14; (lane 14) S. typhimurium χ4072 with pYA1078. The immunologically reactive proteins specified by λgt11::M. leprae clone L14 are larger in size because they are fusion proteins with β-galactosidase.

The S. typhi strains χ4297, χ4417 and χ4435 with the pYA1077 recombinant vector are candidates to immunize humans to protect against typhoid fever and leprosy. Efficacy of such vaccines will be dependent upon identifying one to several M. leprae antigens that would elicit protective immune responses and having them specified by cloned genes in an Asd⁺ vector in the S. typhi Δcya Δcrp Δcdt Δasd strains which could then be used in human immunization trials.

Example 8

This example provides a procedure for testing the safety, immunogenicity, and efficacy of live oral vaccines comprised of Δcya Δcrp mutants of S. typhi. The strains tested are Δcya Δcrp derivatives of Ty2, ISP1820 and ISP2822.

The Individuals Studied. The individuals studied are volunteers who are healthy adult humans aged 18-39 years. The prospective volunteers are screened before the study. The screening procedure includes:

1. medical history
2. physical examination
3. electrocardiogram
4. urinalysis
5. complete blood count
6. blood chemistries (BUN, creatinine, fasting blood glucose
7. Serum Na^+, Cl^-, K^+, HCO_3^-
8. VDRL
9. Hepatitis B surface antigen

10. HIV antibody by ELISA
11. Pregnancy test (females)
12. Liver function tests (SPGT)
13. Psychological examination and interviews.

The Volunteers to participate in the study are selected on the basis of general good health and have:

1. no clinically significant history of gall bladder disease, immunodeficiency, cardiovascular disease, respiratory disease, endocrine disorder, liver disease including a history of hepatitis, renal and bladder disease, enlarged prostate, glaucoma, gastrointestinal disease, disorder of reticuloendothelial system, neurologic illness, psychiatric disorder requiring hospitalization, drug or alcohol abuse;
2. normal and regular bowel habits falling within the limits defined for a normal population: at least 3 stools per week and less than 3 stools per day without frequent use of laxatives or antidiarrheal agents;
3. absence of allergy to amoxicillin or ciprofloxacin;
4. no history of any antibiotic therapy during the 7 days before vaccination;
5. a negative pregnancy test (females);
6. a negative HIV antibody test.

The Volunteers are admitted to an Isolation Ward, and informed, witnessed, written consent is obtained.

Study design. Groups of 22 volunteers are studied. Baseline blood and intestinal fluid specimens are collected. After a two-day period of acclimatization on the ward, the fasting volunteers are randomly allowed to ingest with bicarbonate buffer a single oral dose containing 5 x 10^9 of either the Δcya Δcrp derivative of Ty2, ISP1820 or ISP2822. The volunteers are observed for the next 15 days for adverse reactions (fever, malaise, chills, vomiting, diarrhea) (the usual incubation period of typhoid fever is 8-12 days). Serial blood and stool

cultures are obtained. In addition, any volunteer who has a temperature elevation to 100.8°F has blood samples drawn at the time the observation is made; if the temperature remains elevated at this level for 12 hours, therapy is initiated with oral amoxicillin (1.0 gram every 6h) and oral ciprofloxacin (750 mg every 12h for 10 days). Duodenal fluid cultures are also obtained during the period of observation on days 7, 10, and 13.

Animal tests. The LD_{50}s for the parent strains and attenuated derivatives in mice by intraperitoneal inoculation with hog gastric mucin as adjuvant are also determined.

Preparation of the vaccine inocula. Stock cultures of the S. typhi candidate vaccine strains are stored as a cell suspension in trypticase soy broth (TSB), supplemented with 15% glycerol, at -70°C until needed. To make an inoculum of each strain, the suspension is thawed and plated onto sheep red blood cell agar (5% srbc in TSA), two days before challenge. After incubation at 37°C overnight, about 20-30 typical colonies are picked and suspended in saline. This suspension is inoculated onto trypticase soy agar plates, appropriately supplemented, and the plates incubated overnight at 37°C. In preparation for orally vaccinating the volunteers, growth on these plates is harvested with approximately 3 ml sterile normal saline per plate. The resulting suspension is standardized turbidimetrically. Dilutions are made in saline to approximate the concentration of Salmonella required. The vaccine inoculum is transported to the isolation ward on ice. Microscopic examination and slide agglutination with S. typhi O and H antisera are performed before use. Replica spread plate quantitative cultures are made of the inocula before and after vaccination to confirm viability and inoculum size.

Inoculation of Volunteers. The vaccine is administered by the oral route with $NaHCO_3$. Volunteers are

NPO for 90 minutes before vaccination. Two grams of $NaHCO_3$ are dissolved in 5 ounces of distilled water. Volunteers drink 4 ounces of the bicarbonate water; one minute later the volunteers ingest the vaccine suspended in the remaining 1 ounce of bicarbonate water. Volunteers take no food or water for 90 minutes after inoculation.

Procedures for Specimen Collection.

Stool specimens. A record is kept of the number, consistence, and description of all stools passed by volunteers. A specimen of every stool (or rectal swab if stool is not passed) is collected for culture. The volume of the specimen is measured. Stools are graded on a five point system:

grade 1-firm stool (normal)
grade 2-soft stool (normal)
grade 3-thick liquid (abnormal)
grade 4-opaque watery (abnormal)
grade 5-rice water (abnormal).

Phlebotomy. Serum for antibody determinations is obtained before and 8, 21, 28, 60, and 180 days after vaccination. Heparinized blood for lymphocyte separations for antibody-secreting cell assays is collected on days 0, 4, 7, and 10. Mononuclear cells collected on days 0, 28, 60, and 180 days are used to assess lymphocyte proliferative responses to Salmonella and control antigens. Lastly mononuclear cells from days 0, 28, 60, and 180 are also used in the antibody-dependant cytotoxicity assay against S. typhi and control organisms. Blood (5 ml) is obtained for culture on days 3, 4, 7, 8, 10, 12, and 15 during the post-vaccination observation period to detect vaccine organisms. An additional specimen of serum and mononuclear cells are obtained 180 days after primary vaccination.

Jejunal fluid aspiration. Before oral vaccination and immediately before discharge (day 15), volunteers swallow polyvinyl chloride intestinal tubes to a distance

of 130 cm from the mouth to collect intestinal fluid for measurement of local SIgA antibody. Ten mg of metoclopramide is given orally after ingestion of the tube to accelerate its passage from the stomach through the pylorus into the small intestine. Placement of the tubes in the jejunum is verified by distance (130 cm), color (yellow-green), and pH (6) of aspirated fluid. Approximately 100 ml of jejunal fluid is removed at each intubation.

Gelatin String Capsules. In order to determine rates of intestinal colonization with each vaccine strain, gelatin string capsules (Entero-Test) are ingested by volunteers three times during the period of hospitalization.

The volunteer is NPO from 6 A.M. A swallow of water is used to moisten the mouth and throat. The capsule, with a portion of the string pulled out, is swallowed with water while holding the loop of the nylon string. The line is secured to the face, and left in place for 4 hours. The volunteers are allowed to drink water ad lib, but are not allowed other food or beverages. After 4 hours, the line is withdrawn, the distal section saturated with bile stained mucus is cut and placed in a sterile petri dish, which is labeled for identification. The strings are then cultured for microorganisms, using the same method as with the stool specimens.

Tonsillar Cultures. In order to detect possible invasion of tonsillar lymph tissue after vaccination, serial tonsillar cultures are obtained on days 3, 4, 7, 8, 10, 12, and 15.

Bacteriological Analysis. Stools, rectal swabs, and the distal 15 cm of bile-stained duodenal string from the ingested gelatin capsule is inoculated into selenite F enrichment broth. Tonsillar swabs are inoculated into GN broth. After overnight incubation at 37°C, subcultures are made onto Salmonella-Shigella agar and XLD agar, both

appropriately supplemented for the auxotrophy of the vaccine strain. Suspicious colonies are transferred to supplemented triple sugar iron slants and confirmation made by agglutination with S. typhi Vi, O, and H antisera. These isolates are saved at -70°C in glycerol for further analysis (e.g., for the presence of plasmids or for Southern blotting with specific gene probes for cloned genes).

Blood cultures (5 ml) are inoculated into 50 ml of supplemented brain heart infusion broth.

Immunological Analysis. Sera and jejunal fluid specimens are tested for IgA, IgM, and IgG antibodies to S. typhi O, H, and Vi antigens measured by ELISA, using the procedures described by Levine et al. (1987), J. Clin. Invest. 79:888-902. H antibody is also measured by Widal tube agglutination using S. virginia as antigen (S. virginia shares an identical flagellar antigen with S. typhi).

Peripheral blood mononuclear cells are collected and separated for studies of specific responses to Salmonella antigens. These include the following.

1. Antibody-secreting cells: trafficking lymphocytes with secrete IgG, IgA or IgM antibody against S. typhi O, Vi or H antigens are measured by the method of Kantele et al.

2. Replicating lymphocytes: peripheral blood mononuclear cells are mixed with heat-phenol-activated S. typhi, S. typhimurium, S. thompson, and E. coli to detect antigen-driven lymphocyte replication, as described in Levine et al., supra.

3. ADCC: plasma-mediated mononuclear cell inhibition of S. typhi is measured in an antibody dependent cellular cytotoxicity assay as described in Levine et al., supra.

Excretion of the Vaccine Strain. It is expected that excretion of the vaccine strain would cease within 1

week after a dose of vaccine. If excretion continues for 7 or more days, the volunteer who continues to excrete is given a dose of ciprofloxacin (750 mg every 12 hours). Negative cultures for ≥2 consecutive days are required for discharge.

Example 9

This example demonstrates the safety and immunogenicity of a Δcya Δcrp S. typhi strain, χ3927, which was prepared from the wild-type parent strain, Ty2. The LD_{50} in mice of this strain is 1.8 x 10^4 (using an intraperitoneal injection with hog gastric mucin).

The procedure followed was essentially that described in Example 8, supra. Two cohorts of volunteers were used for studies in which different doses of vaccine were given. In the first study, 17 volunteers were randomized in a double-blind fashion; 6 volunteers received 5 x 10^3 cfu of χ3927, the remainder received the same dose of other S. typhi strains. In the second study, 19 volunteers were randomized in a double-blind fashion; 6 volunteers received 5 x 10^4 cfu of χ3927, the remainder received the same dose of other S. typhi strains. Volunteers were closely monitored on an Isolation Ward for 15 days (first study) or 24 days (second study). Vital signs were measured every six hours during the period of observation. All stools from each volunteer were collected in plastic containers, examined, graded on a five-point scale, and the volume measured if the stool was loose. Volunteers were interviewed daily by a physician and asked about symptoms. Fever was defined as oral temperature ≥ 38.2°C; diarrhea was defined as two or more loose stools within 48 hours totalling at least 200 ml in volume or a single loose stool ≥ 300 ml in volume. Antibiotic therapy was given to volunteers who developed fever or positive blood cultures.

In order to prepare the vaccine, stock cultures of χ3927 which had been maintained on trypticase soy broth

with 15% glycerol at -70°C were thawed and grown on supplemented aro agar. After incubation at 37°C, 20-30 typical colonies of the vaccine strain were picked from aro agar, suspended in saline, and inoculated again onto aro agar. After overnight incubation at 37°C, the bacteria were harvested with 2 ml of sterile phosphate buffered saline (PBS) and the concentration of bacteria was standardized turbidimetrically. Dilutions of the suspensions were made in PBS to achieve the desired concentration of viable organisms per milliliter. The identity of the inoculum was confirmed by microscopic examination and by side agglutination with S. typhi O, H, and Vi antisera. Replica spread plate quantitative cultures were made of the inocula before and after vaccination to confirm viability and the inoculum size.

The vaccine strains were administered by the oral route with sodium bicarbonate. Sodium bicarbonate (2 gm) was dissolved in 150 ml of distilled water and volunteers drank 120 ml to neutralize gastric acid. One minute later, volunteers drank the vaccine suspended in the remaining 30 ml of bicarbonate solution. Volunteers had nothing to each or drink for 90 minutes before and after vaccination.

Every stool passed by volunteers (and rectal swabs if no stool was passed) was cultured daily for the vaccine strain. Stool was inoculated into gram Negative broth (BBL, Cockeysville, MD) supplemented with 0.1% PABA and 0.1% PHB and directly onto S-S agar with supplements. After incubation overnight at 37°C, subcultures were made onto supplemented S-S agar. To quantitate the shedding of vaccine strains, 1 g of stool was serially diluted 10-fold in saline and each dilution was plated onto S-S agar supplemented as above. Suspicious colonies were transferred to triple sugar iron agar slants and the identity confirmed by agglutination with S. typhi O, H, and Vi antisera.

On days 7, 10, and 13 after vaccination, fasting volunteers swallowed gelatin capsules containing string devices to collect samples of bile-strained duodenal fluid. After 4 hours, the strings were removed and the color and pH of the distal 15 cm were recorded. Duodenal fluid was squeezed from the end of the string and cultured as above.

Blood for culture of the vaccine organisms was systematically collected on days 4, 5, 7, 8, 10, 12, and 15 after vaccination and again if fever occurred. Five ml of blood was inoculated into 50 ml of supplemented aro broth.

In addition, tonsillar cultures were obtained on days 1, 2, 4, 5, 7, 8, 10, 12 and 15 to detect the vaccine strain. Swabs applied to the tonsils were inoculated into Gram Negative broth with supplements for 24 hours and then onto supplemented salmonella-shigella agar.

In order to determine the immunological response, the following procedures were followed. Serum samples were obtained before and on days 7, 21, 28, and 60 after vaccination. Jejunal fluids were collected before and on day 14 after vaccination, as described in Example 8. The total IgA content of the fluids were measured by ELISA and each specimen was standardized to contain 20 mg of IgA per 100. Antibodies to S. typhi lipopolysaccharide (LPS), H, and Vi antigens were measured in serum and jejunal fluids.

IgG antibody to LPS O antigen was detected by ELISA. A rise in net optical density ≥ 0.20 between pre- and post-vaccination sera tested at a 1:100 dilution was considered a significant rise. The positive control serum used with each microtiter plate contained a high level of LPS O antibody and represented a pool of sera from 12 healthy Chileans who had strong IgG LPS O antibody responses after immunization with Ty21a vaccine. IgA antibody to LPS O antigen was measured using two-fold dilutions of serum, starting with a 1:25 dilution. An IgA titer was considered significant if a 4-fold rise occurred between pre-and post-vaccination procedures.

Intestinal secretory IgA antibody to S. typhi LPS O antigen was also measured by ELISA. Four-fold rises were considered significant.

In order to measure H antibody, H-d flagellar antigen was prepared from S. typhi strain 541 Ty. Serum and jejunal fluid for H-d antibody was measured by ELISA. A 4- fold rise in titer was considered significant.

The Widal tube agglutination test for H antibody was performed using Salmonella virginia which shares the flagellar antigen d with S. typhi, but no other antigens.

Vi antibody was measured in serum and jejunal fluid by ELISA; a 4-fold rise was considered significant.

Gut-derived, trafficking antibody secreting cells (ASC) that secrete IgG, IgA, or IgM antibody against S. typhi O, H, or Vi antigens were measured by a modification of the method of Forrest et al. ((1988), Lancet 1:81) using both ELISA and ELISPOT assays. Heparinized blood was drawn before and on days 7 and 10 after vaccination. Briefly, peripheral blood lymphocytes separated by a Ficoll gradient (Organon Teknika, Durham, NC) were added to antigen-coated plates. In the ELISA, binding of antibody secreted by lymphocytes was measured by the change in optical density produced by the reaction of the substrate with bound anti-IgA conjugate. Significant responses to LPS, H, and Vi antigens were determined using the differences in O.D. plus 3 S.D. generated from pre-immunization and day 4 cells taken from volunteers participating in these studies. In the ELISPOT assay, specific IgA secreted by individual lymphocytes was detected by adding an agarose overlay to each well and counting colored spots produced by reaction of the substrate with bound ant-human IgA conjugate. Detection of ≥ 4 spots per well after vaccination was defined as a positive response; this number is based on the mean number of spots counted before vaccination plus 2 S.D.

The results obtained were the following.

The clinical signs and symptoms of volunteers after vaccination were evaluated in a double-blind fashion. One of 12 volunteers who received strain χ3927 had fever. This volunteer developed fever with a maximum temperature of 40.1°C on day 22 after vaccination. This volunteer had severe abdominal cramps, malaise, anorexia, headache, and vomiting on days 4-13, but his fever did not being until day 22. His symptoms then included dizziness, muscle and body aches, constipation, insomnia, and cough productive of brown sputum. Another volunteer in this group had malaise, cramps, headache, and nausea during the inpatient surveillance period.

The bacteriology studies showed that one of six volunteers who received 5 x 10^4 and one of six volunteers who received 5 x 10^5 cfu of χ3927 had positive blood cultures. These occurred on days 15 and days 8 and 12, respectively. Neither of these volunteers had any symptoms. One of the 12 volunteers who received χ3927 had one colony of vaccine organisms detected in the stool on day 1. None of these volunteers had positive tonsillar or duodenal string cultures. The χ3927 isolates recovered from the blood and the stool of volunteers retained all expected phenotypes associated with the presence of Δcya Δcrp mutations.

The immunological studies show that six (50%) of the 12 vaccines who received χ3927 developed IgG anti-S. typhi LPS responses. No antibody to H antigen or Vi were detected in any of the twelve volunteers. Only one of the twelve volunteers developed secretory IgA against LPS in the jejunal fluid. Secretory IgA antibody responses to H antigen occurred in only one volunteer and no volunteer had secretory anti-Vi antibody after vaccination. Five of 12 volunteers developed circulating cells secreting IgA against LPS detected by ELISA or ELISASPOT assay.

The degree of attenuation conferred by deletions in the cyclic AMP regulatory pathway cannot be strictly

measured without simultaneous challenge of volunteers with mutant and parent strains. However, based on historical experience with volunteers given similar doses of wild type strains, it is likely that the deletions confer attenuation to S. typhi. When wild-type S. typhi strain Ty2 was fed to six volunteers at a dose of 1 x 10^7 without bicarbonate, 83% developed typhoid fever (defined as temperature 103°F for >36 hours) or infection (defined as low grade fever, significant serologic response, positive blood culture, or excretion of S. typhi for > 5 days. In contrast, among the 12 volunteers reported herein who received the χ3927 vaccine derived from Ty2 at a dose of 10^4 or 10^5 cfu with bicarbonate (equivalent to a much higher dose without bicarbonate), fever occurred in only one volunteer and positive blood cultures in only two volunteers. Moreover, volunteers who had febrile illnesses did not have vaccine bacteria detected in their blood, despite additional blood cultures collected at the time of fever. It is likely that fever occurred in response to the release of cytokines stimulated by the enteric infection with the vaccine.

Example 10

This example describes the construction and characterization of Δcrp-10 Δcya-12 S. typhi constructs which contain a Δcdt mutation. We have introduced Δcya Δcrp mutations into S. typhi Ty2 (type E1) and S. typhi ISP1820 (a Chilean epidemic type 46 isolate). The former strain with Δcya-12 and Δcrp-11 mutations has already been evaluated in human volunteers, described in Example 9. One of six volunteers who received 5 x 10^4 cfu and one of six volunteers who received 5 x 10^5 cfu of the Δcrp-11 Δcrv-12 S. typhi strain, χ3927, had positive blood cultures. These occurred on day 15 and days 8 and 12, respectively. However, neither of these volunteers had any symptoms. Furthermore, not all immunized individual developed high-titer antibody responses to S. typhi antigens. Additional attenuating mutations which would permit higher oral doses

for induction of protective immunity in the majority of those immunized, are desirable. We have identified an additional gene defect that has been introduced into Δcya Δcrp S. typhi strains that results in decreased virulence and should thus permit higher dosages. The defect is a deletion in a gene termed cdt for colonization of deep tissues. Strains with a Δcdt mutation, in addition to Δcya and Δcrp mutations are also less able to survive in human serum than are strains with only Δcya Δcrp mutations. They should therefore be cleared more readily and would be less likely to induce vaccinemia.

Strain construction

The wild-type, virulent S. typhi Ty2 (Type E1) and ISP1820 (Type 46) strains have been genetically modified using classical genetics by similar methods described in Curtiss and Kelly ((1987), Infect. Immun. 55:3035-3043), and described in Example 1. Salmonella typhimurium deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect. Immun. 55:3035-3043.(1). The strategy consists of facilitating transduction of deletions of crp-cdt (designated Δcrp-10) and cya genes that have been isolated and characterized in S. typhimurium SL1344 by placing the transposon Tn10 (encoding tetracycline resistance) nearby the cya or crp deletion. We have therefore used zhc-1431::Tn10 linked to Δcrp-10 and zid-62::Tn10 linked to Δcya-12, respectively, and cotransduced with P22HTint the linked traits into the highly virulent S. typhi Ty2 and ISP1820 strains with selection for tetracycline resistance and screening for a maltose-negative phenotype.

Transduction of the gene deletion with the linked transposon was facilitated by first making a high-titer bacteriophage P22HTint lysate on an S. typhimurium strain χ3712 containing the Δcrp-10 zhc-1431::Tn10 mutations and another lysate on an S. typhimurium strain χ3711 containing the Δcya-12 zid-62::Tn10 mutations. The resulting P22HTint

lysates were then used to infect and transduce the genetic traits into the recipient S. typhi Ty2 (χ3769) and ISP1820 (χ3744) strains at a multiplicity of infection of 10.

P22HTint propagated on S. typhimurium χ3712 (Δcrp-10 zhc-1431::Tn10) was used to transduce the virulent S. typhi Ty2 and ISP1820 strains to Mal⁻ Tet^r. The phage-bacteria infection mixture was incubated for 20 min at 37°C before 100 μl samples were spread onto MacConkey agar (Difco Laboratories, Detroit, MI) containing 1% maltose (final concentration) supplemented with 12.5 μg tetracycline/ml. After approximately 26-36 h incubation at 37°C, transductants were picked and purified onto the same media. The resulting Ty2 derivative was designated χ3792 and the ISP1820 derivative was designated χ4324. Both have the genotype Δcrp-10 zhc-1431::Tn10. Strains χ3792 and χ4324 were grown in Luria broth[1] + 12.5 μg tetracycline/ml and each were diluted 1:10 into buffered saline with gelatin (BSG). Samples of 100 μl of each strain were spread onto fusaric acid-containing (FA) media (Maloy and Nunn (1981); J. Bacteriol. 145:1110-1112) and the plates incubated approximately 36 h at 37°C. Fusaric acid-resistant colonies of each strain were picked into 0.5 ml BSG and purified by streaking onto FA media. Purified fusaric acid-resistant colonies were picked into Luria broth and grown at 37°C to turbidity and checked for loss of Tn10 (tetracycline sensitivity), complete LPS, Vi antigen and auxotrophy for arginine, cystaine and tryptophan. The new strains were designated χ3803 (Ty2) and χ4325 (ISP1820) which have the genotype Δcrp-10 Δ[zhc-1431::Tn10].

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[1] Luria broth contains 10 g of NaCl per liter whereas Lennox broth contains 5 g of NaCl per liter. It has been shown that Salmonella cells grown in high osmolarity media display an increased ability to invade tissue culture cells (Galan and Curtiss, Infect. Immun. (1990) 58:1879-1885; expression of Salmonella genes required for invasion is regulated by changes in DNA supercoiling). Therefore, the increased NaCl level in Luria broth ensures optimal effectiveness of the vaccine strain.

Since the phenotype of Cya⁻ and Crp⁻/Cdt⁻ mutants are the same (Mal⁻, Stl⁻, Mtl⁻, etc.), the plasmid, pSD110, carrying the cloned wild-type crp⁺ gene with its promoter (Schroeder and Dobrogosz (1986), J. Bacteriol. 167:616-622.) was used to temporarily complement the Δcrp mutation in the chromosome (thus restoring the strain to the wild-type phenotype) and enabling the identification of strains with the Δcya mutation after transduction. Luria broth cultures of χ3803 and χ4325 were transduced with P22HTint propagated on S. typhimurium χ3670, which contains the plasmid pSD110. Selection was made on MacConkey agar + 1% maltose + 100 μg ampicillin/ml. After 26 h, an ampicillin-resistant, Mal⁺ colony of each strain was picked and purified on MacConkey agar + 1% maltose agar and designated χ3824 (Ty2) and χ4331 (ISP1820) which have the genotype Δcrp-10 [zhc-1431::Tn10] pSD110⁺.

Strains χ3824 and χ4331 were grown in L broth + 100 μg ampicillin/ml and were each independently transduced with P22HTint propagated on χ3712 to introduce the Δcya-12 and the linked zid-62::Tn10 mutations. Selection for a maltose negative, tetracycline resistance, ampicillin resistance phenotype was made on MacConkey agar + 1% maltose + 100 μg ampicillin/ml + 12.5 μg tetracycline/ml. Ampicillin-resistant (pSD110⁺), tetracycline-resistant (zid-62::Tn10), Mal⁻ (Δcya) colonies were picked and purified onto MacConkey agar + 1% maltose + 100 μg ampicillin/ml + 12.5 μg tetracycline/ml. Purified colonies were picked into Luria broth, grown to turbidity and the strains checked for complete LPS, Vi antigen and auxotrophy for arginine, cysteine and tryptophan. Isolates of the correct phenotype were designated χ3919 (Ty2) and χ4340 (ISP1820) which have the genotype Δcrp10 Δ[zhc-1431::Tn10] pSD110⁺ Δcya-12 zid-62::Tn10. Cultures of χ3919 and χ4340 were grown in L broth + 100 μg ampicillin/ml + 12.5 μg tetracycline/ml to turbidity, diluted 1:10 into BSG, and 100 μl samples of each culture spread onto fusaric-

containing media and incubated approximately 36 h at 37°C. Fusaric acid-resistant colonies of each strain were picked and purified onto FA media. Purified FA-resistant colonies were picked into Luria broth, grown to turbidity and then checked for loss of Tn10 (tetracycline sensitivity), complete LPS, Vi antigen and auxotrophy for arginine, cysteine and tryptophan. The pSD110 plasmid was spontaneously lost during growth of the strains in the absence of ampicillin. The final strains which were ampicillin-sensitive and plasmid-free were designated χ3924 (Ty2) and χ4345 (ISP1820) which have the genotype Δcrp-10 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10]. Since synthesis of flagella with display of motility is partially dependent upon functional cya and crp genes and since flagella are important antigens, we selected derivatives of χ3924 and χ4346 that possess a suppressor mutation (cfs) that permits flagella synthesis and function to be independent of the cya and crp gene functions. χ4073 was selected as a flagella-positive derivative of χ3924, and χ4346 was selected as a flagella-positive derivative of χ4345. Table 3 lists the wild-type parent strains and their Δcya Δcrp derivatives.

Strains χ4073 and χ4346 can easily be distinguished from their wild-type parents by the following phenotypic characteristics: the inability to ferment or grow on the carbon sources maltose, mannitol, sorbitol, melibiose and xylose, inability to produce H_2S, increased generation time, and the significantly increased murine LD_{50} values.

Table 10

Bacterial Strains

χ3769, S. typhi Ty2

Type E1, wild type, Vi⁺.

Received from L. Baron, Walter Reed Army Institute of Research, Washington, DC, as Ty2.

χ4073 S. typhi Ty2

Δcrp-10 [zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10]; Crp⁻ Cdt⁻ Cya⁻ Arg⁻ derivative of χ3769.

χ3744 S. typhi ISP1820

Type 46, wild type, Vi⁺.

Received from M. Levine, Center for Vaccine Development, Baltimore, MD, as ISP1820. 1983 isolate from a Chilean patient.

χ4346 S. typhi ISP1820

Δcrp-10 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10]; Crp⁻ Cdt⁻ Cya⁻ Arg⁻ derivative of χ3744.

Growth conditions for χ3744, χ3769, χ4073 and χ4346

Cells of each strain were picked from agar medium into 2 ml Luria broth. Cultures were incubated as static cultures at 37°C for approximately 14 h. When the cultures were visibly turbid ($OD_{600} \geq 0.5$), a loopful of each culture was streaked for isolated colonies on the media listed in Table 11 to verify some of the phenotypic properties. Cultures were also tested for sensitivity to phages, antibiotic susceptibility, ability to produce wild-type LPS, auxotrophy, motility, inability to produce colicins, absence of plasmid DNA, mean generation time, and agglutination by antisera to identify the O, H and Vi antigen of S. typhi (see Table 11). The phenotypic properties of all strains were as expected with the Δcya Δcrp strains χ4346 and χ4073 growing significantly more slowly than their wild-type parents.

Table 11

Phenotypic characterization of S. typhi wild-type and Δcrp-10 Δcya-12 strains

	Phenotype			
	χ3744	χ4346	χ3769	χ4073
MacConkey Base Agar +				
1% maltose	+	-	+	-
1% sorbitol	+	-	+	-
1% mannitol	+	-	+	-
1% melibiose	+	-	+	-
1% rhamnose	-	-	-	-
1% citrate	-	-	-	-
1% arabinose	-	-	-	-
1% mannose	+	+	+	+
1% xylose	+	-	+	-
1% glucose	+	+	+	+
Minimal agar* +				
0.5% glucose	+	+	+	+
0.5% sorbitol	+	-	+	-
0.5% mannitol	+	-	+	-
0.5% melibiose	+	-	+	-
0.5% rhamnose	-	-	-	-
0.5% citrate	-	-	-	-
0.5% arabinose	-	-	-	-
0.5% mannose	+	+	+	+
0.5% xylose	+	-	+	-

*minimal media recipe attached; supplements include L-arginine HCl 22 μg/ml, L-cysteines HCl 22 μg/ml, L-tryptophan 20 μg/ml.

	Phenotype			
	χ3744	χ4346	χ3769	χ4073
Triple Sugar Iron media - H_2S production	+	-	+	-
alkaline slant -	Lac⁻ Glu⁺ Suc⁻	Lac⁻ Glu⁺ Suc⁻	Lac⁻ Glu⁺ Suc⁻	Lac⁻ Glu⁺ Suc⁻
Indole fermentation assay	-	-	-	-
Bacteriophage sensitivity				
ViII	S	S	S	S
Felix-O	S	S	S	S
p22HTint	S	S	S	S

Table 11 (cont'd)

	P1L4	R	R	R	R
	L	R	R	R	R
	KB1	R	R	R	R
LPS profile by SDS-PAGE (silver stain) (Comp. = complete)		comp	comp	comp	comp
Motility		+	+	+	+
Colicin(2) production		-	-	-	-

[a] phage sensitivity was assayed by soft agar overlay technique of by transduction. S = sensitive; R = resistant.

[d] Motility determined by stabbing a loopful of a standing-overnight Luria broth culture into media containing 1.0% casein, 0.5% NaCl, 0.5% Difco agar and 50 μg/mg triphenyl-tetrazolium chloride; incubation at 37°C and motility recorded at 24 and 48 h.

	Phenotype			
	χ3744	χ4346	χ3769	χ4073
MGT[a]	26.6	26.6	26.6	26.6
Plasmid content	none	none	none	none
Auxotrophy	Cys⁺ Trp⁺ Arg⁺	Cys⁺ Trp⁺ Arg⁺	Cys⁺ Trp⁺ Arg⁺	Cys⁺ Trp⁺ Arg⁺
MIC[f]				
Tetracycline	4	4	<2	4
Streptomycin	64	64	16	8
Ampicillin	<2	<2	<2	<2
Gentamicin	<2	<2	<2	<2
Chloramphenicol	4	4	4	4
Neomycin	<2	<2	<2	<2
Rifampicin	8	16	8	8
Nalidixic acid	<2	4	<2	4
Spectinomycin	32	32	32	16
Kanamycin	<2	<2	<2	<2

Table 11(cont'd)

[a] Mean Generation Time (min.) = determined in Luria broth with aeration (150 rpm New Brunswick platform shaker) at 37°C.

[f] Minimal Inhibitory Concentrations (μg/ml) of antibiotics were determined by streaking standing-overnight cultures of each strain onto agar containing defined concentrations of antibiotics.

	Phenotype			
	χ3744	χ4346	χ3769	χ4073
Agglutination with Difco antisera to:				
flagellar antigen H:1	+	+	+	+
flagellar antigen H:2	+	+	+	+
Group D factor 9	+	+	+	+
Group D factor 12	+	+	+	+
Group D (O-1,9,12)	+	+	+	+

Growth characteristics on agar media

Strains were grown in Luria broth as standing-overnight cultures at 37°C, diluted in buffered saline and gelatin (BSG) and plated on MacConkey agar containing 1% maltose to achieve isolated colony-forming units (cfu). All colonies of a given strain appear uniform in size and color. Due to the slower growth rates of Δ*cya crp* strains compared to their wild-type parents, growth on MacConkey media takes ~36 h at 37°C before colonies of χ4073 and χ4346 are easily visible.

Stability of mutant phenotypes

Fifty-fold concentrated cultures and various dilutions (~ 10^9, 10^7, 10^5, 10^3 cfu/plate) of χ4073 and χ4346 were plated on minimal agar media (supplemented with 22 μg L-arginine/ml, 22 μg L-cysteine/ml and 20 μg L-tryptophan/ml) containing either 0.5% maltose, melibiose, xylose, glycerol, or rhamnose that should not support their growth. One set of duplicate plates were UV-irradiated (5 joules/meter^2/sec) and incubated at 37°C in the dark. The

other set was incubated at 37°C with illumination. No revertants and/or mutants were detected after a 48 h growth period.

Storage of strains

Each strain was maintained in a 1% peptone-5% glycerol suspension and stored at -70°C.

Preparation of inoculum for animal experimentation

The following is a standardized protocol for growth and suspension of each vaccine strain and its parent for intraperitoneal (i.p.) inoculation of mice.

Female CFW mice (18-29 g) (Charles River, Wilmington, MA) were used for determining LD_{50} values of wild-type S. typhi and virulence of the Δcrp-10 Δcya-12 derivatives. Static overnight cultures (37°C) were diluted 1:20 into prewarmed Luria broth and aerated (150 rpm) at 37°C until an OD_{600} of ≤ 0.08 was reached. Wild-type and Δcrp-10 Δcya-12 S. typhi cells were suspended in 15% (wt/vol) hog gastric mucin (American Laboratories, Omaha, NB). The 15% mucin suspension was prepared by neutralizing to pH 7, autoclaving 10 min at 121°F at 15 p.s.i., and 3 μg of freshly prepared sterile ferric ammonium citrate/ml (Sigma, St. Louis, MO) was added prior to adding appropriately diluted S. typhi cells. The cell suspensions were then administered i.p. to CFW mice through a 23-gauge needle in 500 μl volumes. LD_{50} values of the wild-type parents and the Δcrp-10 Δcya-12 derivatives were determined after recording mortality data for 72 h. See Table 12 for results on virulence of S. typhi mutants relative to wild-type parents.

Table 12. Virulence of ISP1820 and Ty2 S. typhi wild-type and Δcrp-11 Δcrp-10 strains

Strain No.	Genotype	LD_{50}[1] CFU
χ3744	ISP1820 wild type	32
χ4299	Δcrp-11 Δ[zhc-1431::Tn10]	<600
χ4300	Δcrp-11 [zhc-1431::Tn10]/ pSD110*[2]	107
χ4323	Δcrp-11 Δ[zhc-1431::Tn10] Δcya12 Δ[zid-62::Tn10]	>2.8 x 10^3
χ4325	Δcrp-10 Δ[zhc-1431::Tn-10]	>3.2 x 10^6
χ4331	Δcrp-10 Δ[zhc-1431::Tn10]/ pSD110*	>2.3 x 10^6
χ4346	Δcrp-10 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10]	4.4 x 10^3
χ3769	Ty2 wild type	54
χ3878	Δcrp-11 Δ[zhc-1431::Tn10]	1.0 x 10^3
χ3880	Δcrp-11 Δ[zhc-1431::Tn10] pSD110*	<19
χ3927	Δcrp-11 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10]	1.1 x 10^4
χ3803	Δcrp-10 Δ[zhc-1431::Tn10]	1.5 x 10^5
χ3824	Δcrp-10 Δ[zhc-1431::Tn10]/ pSD110*	>1.9 x 10^5
χ4073	Δcrp-10 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10]	>1.0 x 10^6

[1] LD_{50} calculated by method of Read and Muench (1938. Am. J. Hyg. 27:493-497.) Morbidity and mortality data collected over a 72 h period.

[2] pSD110 (Schroeder, C.J., and W.J. Dobrogosz. 1986. J. Bacteriol. 167:616-622 is a pBR322 derivative containing the wild-type crp⁺ gene and its promoter from S. typhimurium. Previous virulence assays have shown this

plasmid to complement a crp mutation in S. choleraesuis, S. typhimurium and S. typhi and restore virulence to wild-type levels.

Mammalian cell culture adherence and invasion assays

Data on the ability of Δcrp-10 Δcya-12 and Δcrp-11 Δcya-12 strains to adhere to and invade CHO cells as compared to the wild-type parent strains are presented in Table 13. The S. typhi mutants show a reduced capability to adhere to and/or invade monolayers to CHO cells over a 2-h and 4-h period, respectively, at 37°C as compared to the wild-type parent strains.

Table 13. Adherence and invasion of CHO cell monolayers by S. typhi wild-type and Δcrp Δcya strains

Strain No.	Genotype	Percent adherence[1]	Percent invasion[2]
χ3744	wild type	43.5-6.5	34.2-8.3
χ4323	Δcrp-11 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10]	20.8-1.6	8.3-0.4
χ4346	Δcrp-10 Δ[zhc-1431::Tn10] Δcya-12 Δ[zid-62::Tn10]	8.3-0.7	5.3-2.2

[1] Percentage of inoculum adhered to cells after incubation for 2 h.

[2] Percentage of inoculum recovered from CHO cells 2 h after incubation in 100 μg gentamicin/ml.

Values are mean - SD of triplicate samples.

Growth and persistence of mutants in normal human sera as compared to wild-type parents

Growth curves were performed in normal human sera that has previously been adsorbed with wild-type S. typhi. Approximately 10^4 cfu of S. typhi Δcya Δcrp and wild-type strains were added to each ml of sera that had been equilibrated with HEPES at 37°C in a 5% CO^2 chamber.

Complement-mediated bacteriolysis activity was verified by inactivating sera at 60°C for 10 min and checking growth of E. coli K-12 after 60 min. In normal sera, E. coli K-12 cells were killed in sera after 60 min.

More specifically, χ3744 (ISP1820, wild type), χ3769 (Ty2, wild type), χ4073 (Ty2 Δcya-12 Δ[crp-cysG1 - 10), χ4346 (ISP1829 Δcya-12 Δ[crp-cysG - 10), and χ289 (E. coli K-12) were grown in Luria broth as standing overnight cultures at 37°C. Human serum was adsorbed with the homologous wild-type S. typhi Ty2 and ISP 1820 strains χ3769 and χ3744, respectively, buffered with 20 mM HEPES and incubated in a 5 CO_2 atmosphere for assays. The E. coli K-12 χ289 strain represented a positive control for complement mediated bacteriolysis and the same strain when grown in heat-inactivated serum served as the negative control as is evident by net growth.

Example 11

This example describes the preparation, expression and immunogenicity of internally fused DNA constructs comprised of hybrid HBcAg/Plasmodium circumsporozoite (CS) repeat sequences in Salmonella.

The hybrid HBc/CS genes were constructed by insertion of synthetic oligonucleotides into the HpaI and XbaI sites of the HBcAg gene which was inserted in the prokaryotic expression vector pNS14PS2 which is described in Schodel et al., Vaccines 91, 319-325 (1991). The insertion site is an internal position of the HBc molecule which is surface accessible and highly immunogenic for inserted heterologous epitopes. The structure of the HBc-CS inserts and the location of the CS repeats for P. falciparum and P. berghei in pC75CS2 and PC75CS1 are shown in Figure 2. The amino acid sequence positions of the HBc-CS gene expression products are indicated starting with the HBcAg methionine. The CS repeat sequences derived from the P. berghei and P. falciparum circumsporozoite proteins are indicated in the single letter amino acid code. A sequence

derived from the hepatitis B virus pre-S2 sequence is fused to the C-terminus of the expression products (Schodel et al., J. Virol. 66:106-114, 1992). The oligonucleotide sequences used for construction of pC75CS1 which contains the [(DP$_4$NPN)$_2$] repeat sequence of P. berghei and pC75C2S which contains the [(NANP)$_4$] repeat sequence of P. falciparum are set forth below:

(NANP)$_4$ 1:5'-AAC GCT AAC CCG AAT GCT AAC CCG AAC GCT AAC CCG AAC GCT AAC CCG-3' (SEQ ID NO 1);

(NANP)$_4$ 2:5'-CTA GAC GGG TTA GCG TTC GGG TTA GCG TTC GGG TTA GCA TTC GGG TTA GCG TT3' (SEQ ID NO 2);

(DP$_4$NPN)$_2$ 1:5'-GAC CCG CCG CCG CCG AAC CCG AAC GAC CCG CCG CCG CCG AAC CCG AAC T - 3' (SEQ ID NO 3);

(DP$_4$NPN)$_2$ 2:5'-CTA GAG TTC GGG TTC GGC GGC GGC GGG TCG TTC GGG TTC GGC GGC GGC GGG TC-3' (SEQ ID NO 4).

Oligonucliotides (NANP)$_4$1 and (NANP)$_4$2 are complementary and include a XbaI sticky and for insertion and ligation. Similarly, oligonucleotides (DP$_4$NPN)$_2$1 and (DP$_4$NPN)$_4$2 are complimentary and include a XbaI sticky and for insertion and ligation. The complementary oligonucleotides were annealed prior to insertion into the vectors. Sequances of the vectors were verified by dideoxy DNA sequencing and the expression products verified by incubation with a polyclonal mouse serum directed against P. berghei CS (anti-P.B.) (provided by Dr. Dan Gordon), a monoclonal antibody directed against the P. falciparum CS repeat region (anti-P.F.) (F2A10, provided by Dr. B. Wirtz) and a monoclonal antibody against hepatitis B virus pre-S2 (anti-pre-S2) (448 provided by M. Mayumi). Bound antibodies were visualized on X-ray film using goat anti-mouse IgG (H+L) HRPO (Caltag, South San Francisco, CA) and enhanced chemiluminescence (ECL, Amersham).

The expression vectors pC75CS1 and pC75CS2 were purified from their E. coli hosts and moved into avirulent Δcya Δcrp S. typhimurium χ4064. Synthesis of the hybrid HBc/CS genes in Salmonella typhimurium χ4064 was verified by Western blotting, as shown in Figure 9.

The HBc/CS hybrid gene region has also been inserted into vectors pYBC75CS1, pYNC75CS1 and pYNC75CS2. Plasmid maps of pYBC75CS1 and pYBC75CS2 are provided in Figure 10 and Figure 11, respectively. Plasmid pYBC64CS1 is obtained by ligating the 388 bp PstI-HindIII fragment of pC75CS1 into the PstI-HindIII sites of pYA3167. Plasmid PYBC75CS2 is obtained in a similar manner by ligating the 388 bp PstI-HindIII fragment of pC75CS2 into the PstI-HindIII sites of pYA3167.

The characteristics of these strains are set forth below:

χ4550(pYNC75CS1) S. typhimurium Δcrp-1 Δcya-1 ΔasdA1 with p15a-based HBc/CS from P. berghei Asd$^+$ vector

χ4550(pYNC75CS2) S. typhimurium Δcrp-1 Δcya-1 ΔasdA1 with p15a-based HBc/CS from P. falciparum Asd$^+$ vector

χ4550(pYBC75CS2) S. typhimurium Δcrp-1 Δcya-1 ΔasdA1 with pBR-based HBc/CS from P. falciparum Asd$^+$ vector

χ4064(pC75CS2) S. typhimurium Δcrp-1 Δcya-1 with HBc/CS from P. falciparum

The immunogenicity of χ4064 (pC75CS1) and χ4064 (pC75CS2) were tested by immunizing female BALB/c mice orally once with approximately 2 x 10^9 cfu recombinant S. typhimurium vaccine strains as indicated in Table 14 (cfu were determined by plating of the serially diluted vaccine inoculum on LB agar plates). Pooled sera of five animals/group taken six weeks after immunization were analyzed for IgG antibodies reactive with a synthetic CS repeat peptide Leu-Arg-(NANP)$_{3}$ and S. typhimurium LPS

(Sigma) as solid phase reagents by ELISA. Reciprocal serum dilutions yielding an OD_{490} of 3X that of pre-immune sera are indicated as titers.

TABLE 14

IMMUNOGEN	DOSE (CFU)	TITER (1/) LPS	NANP
χ4064(pC75CS2)	2.7 x 10^9	51,200	51,200
χ4550(pYNC75CS2)	1.2 x 10^9	25,600	25,600
χ4550(pYBC75CS2)	2.6 x 10^9	6,400	25,600
χ4550(pYBC75CS1)	1.9 x 10^9	25,600	<100

As shown in Table 14, a single oral immunization with χ4064 (pC75CS2) or χ4550 (pYNC75CS2 or pYBC75CS2) elicited high titered anti-P. falciparum CS antibodies and immunization with χ4550 (pYBC75CS1) elicited virtually no anti-P. falciparum CS antibodies and served as a negative control. As BALB/c mice are non-responders to CS on a T-cell level, this data implies that non-responsiveness due to MHC restriction can be overcome by using HBcAg core as a carrier moiety when expressed by Salmonella.

Protection against P. berghei challenge

Mice immunized with χ4064(pC75CS1) were analyzed for protection against malarial infections. Control group mice immunized with χ4064(pC75CS2) or χ4064(pNS27-53PS2), and mice immunized with χ4064 (pC75CS1) were infected with P. berghei. For that purpose, *Anopheles stephensi* mosquitos were infected with P. berghei ANKA by feeding on infected mice. Midgut oocyst and salivary gland sporozoite rates were determined to monitor mosquito infections. Mosquitos used for this challenge had a salivary gland sporozoite infection rate of 80% (day 20).

Mice were anesthetized by injection of Rompun:Ketamine and placed on a holding platform after approximately 5 minutes. The mouse tails were laid on top of a screened mosquito container. Mosquitoes were

permitted to feed on a tail until blood was observed in the gut of 5 mosquitoes.

Mice were checked for P. berghei infections after challenge by examination of Geisma-stained thin smear tail bleeds. A minimum of 25 fields per slide (400x) were examined before a mouse was determined negative for infection. Mice were sacrified after 2 consecutive blood smears were obtained.

Four out of five mice orally immunized with χ4064(pC75CS1) were protected against P. berghei challenge (table 15). In the control groups immunized with χ4064(pC75CS2) or χ4064(pNS27-53PS2), both of which express P. falciparum epitopes, four out of five mice developed a parasitaemia when challenged with P. berghei. Those control animals had been immunized with recombinant Salmonella typhimurium which were identical to χ4064(pC75CS1) with the exception of the CS specific epitope. It is therefore reasonable to assume that the higher protection observed in animals immunized with χ4064(pC75CS1) was due to immunity induced by the CS repeat epitope of P. berghei. Immunization with recombinant S. typhimurium by itself may provide a low level of nonspecific protection, which might explain why one out of five animals in the control group was protected. Historically, this route of challenge has repeatedly resulted in a 100% infection take.

Table 15

Immunogen	Serum IgG PB CS	PF CS	Infected/Challenged
χ4064(pC75CS1)	+	-	1/5
χ4064(pC75CS2)	-	+	4/5
χ4064(pNS25-53PS2)	-	-	4/5

Exemple 12

This example illustrates how the pYBC75CS2 vector was moved into a S. typhi strain.

A 5 ml static 37°C overnight Luria broth culture of S. typhi χ4632 (Δcrp-10 Δcya-12 ΔasdA1) was concentrated by centrifugation and the pellet washed once with 100 µl cold 1mM HEPES. The cell pellet was resuspended and washed twice with cold 10% glycerol to a final volume of 40 µl. Plasmid DNA was purified from S. typhimurium χ4550 using the Magic minipreps DNA purification system by Promega. Five microliters of purified DNA was mixed with 40 µl of cold competent cells of χ4632 and placed in cold 0.2 cm cuvette. Electrotransformation was performed at 4°C. The Gene Pulser apparatus was set at 25 µF and the Pulse Controller set at 200 ohms (Bio-Rad, Richmond, CA). The sample was pulsed for 5 msec. Immediately following the pulse, the sample was washed form the cuvette with 1 ml Luria broth and placed in a 13 x 100 mm borosilicate tube and 100 µl plated and spread directly onto MacConkey agar supplemented with 1% maltose. The 1 ml Luria broth electrotransformation mixture was incubated as a static overnight at 37°C and kept as a backup in case the initial plating immediately after pulsing didn't yield any electrotransformants. Three maltose-negative, Asd-positive colonies of χ4632 (pYBC75CS2) were picked and restreaked on fresh MacConkey + maltose media and incubated 37°C overnight. Several colonies of each of the three electrotransformants were checked and the Vi antigen confirmed by agglutination with antisera to Vi antigen (Difco, Detroit, MI). Lipopolysaccharide was analyzed by the methods of Hitchcock and Brown J.Bacteriol. 154:269-277 (1983) and Tsai and Frasch Anal. Biochem. 58:3084-3092 (1982). All three showed LPS profiles the same as the wild-type parent Ty2.

The three independent electrotransformants of χ4632 (pYBC75CS2) were grown in Luria broth 37°C as aerated overnight cultures. The cells were prepared for protein

analysis and subsequant Western blotting by boiling 1 ml of each culture for 5 minutes in 2X SDS/bromophenol blue with B-mercaptoethanol. After centrifugation for 2 minutes, two samples of ten microliters of each sample were electrophoresed each in two 12.5% polyacrylamide separating gels at 200V for one hour. One gel was stained with Coomassie brilliant blue stain (0.1%) to visualize total protein and the other gel was used to electrotransfer the proteins to a nitrocellulose filter. A Western blotting analysis with antisera to the CS2 protein confirmed large quantities of the circumsporozoite protein was expressed by each of the three independent electrotransformants of χ4632.

Deposits of Strains. The following listed materials are on deposit under the terms of the Budapest Treaty, with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland. The accession number indicated was assigned after successful viability testing, and the requisite fees were paid. Access to said cultures will be available during pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 USC 122. All restriction on availability of said cultures to the public will be irrevocably removed upon the granting of a patent based upon the application. Moreover, the designated deposits will be maintained for a period of thirty (30) years from the date of deposit, or for five (5) years after the last request for the deposit, or for the enforceable life of the U.S. patent, whichever is longer. Should a culture become nonviable or be inadvertently destroyed, or, in the case of plasmid-containing strains, lose its plasmid, it will be replaced with a viable culture(s) of the same taxonomic description. The deposited materials mentioned herein are intended for convenience only, and are not required to practice the present invention in view of the description

herein, and in addition, these materials are incorporated herein by reference.

Strain	Deposit DateATCC No.
χ3958	November 2, 1990
55224	
χ4323	November 2, 1990
55115	
χ3926	November 2, 1990
55112	
χ3927	November 2, 1990
55117	
χ4297	November 2, 1990
55111	
χ4346	November 2, 1990
55113	
χ3940	November 2, 1990
55119	
χ4073	November 6, 1991
55248	
ISP2822	November 2, 1990
55114	
ISP1820	November 2, 1990
55116	
χ4417	
55249	
χ4435	
55250	
χ4064 (pNS27-53PS2)	
68959	April 9, 1992
S. typhimurium SR-11	
χ4632 (pYBC75CS2)	April 9, 1993
69278	
χ4550 (pYBC75CS1)	April 9, 1993
69279	

What is claimed is:

1. A composition comprised of live avirulent Salmonella that express at least one recombinant imunogenic antigenic determinant, the antigenic determinant being fused to a Hepatitis B virus core antigen and heterologous thereto.

2. The composition of claim 1 wherein the antigenic determinant is from a Plasmodium species.

3. The composition of claim 2 wherein the plasmodial antigenic determinant is selected from *P. falciparum* or *P. berghei*.

4. The composition of claim 3 wherein the plasmodial antigenic determinant encodes a repeat sequence from the circumsporozoite protein of *P. falciparum* or *P. berghei*.

5. The composition of claim 4 wherein the antigenic determinants are selected from amino acids represented by the nucleotide sequences set forth in SEQ ID NO 1 or SEQ ID NO 2.

6. The composition of claim 2 wherein the plasmodial antigenic determinant is fused at an internal position in the HBV core protein.

7. The composition of claim 6 wherein the plasmodial antigenic determinant is inserted between a first HBV core protein nucleotide fragment coding for amino acids 1-75 and a second HBV core protein protein nucleotide fragment coding for amino acids 81-156.

8. The composition of claim 1 wherein the *Salmonella* is *S. typhi* and the immunogenic antigenic determinant is from *P. falciparum*.

9. The composition of claim 8 wherein the Salmonella is a *cya crp asd* mutant and the antigenic determinant is encoded on a vector encoding Asd.

10. The composition of claim 8 wherein the Salmonella is a *cya crp* mutant.

11. An immunogenic composition comprised of live avirulent Salmonella that express at least one recombinant immunogenic epitope wherein the immunogenic epitope is expressed as a hybrid protein with a region encoding Hepatitis B virus core protein to yield a polypeptide that forms a particle and wherein the immunogenic epitope is heterologous with respect to the Hepatitis B virus.

12. The immunogenic composition of claim 11 wherein the immunogenic epitope is from a Plasmodium species.

13. A method of preparing a vaccine comprising providing a composition comprised of live avirulent Salmonella that express at least one recombinant immunogenic epitope inserted in a Hepatitis B virus core protein, and mixing the composition with a suitable excipient.

14. The method of claim 13 wherein the immunogenic epitope is from a Plasmodium species.

15. A vaccine comprising live avirulent Salmonella that express at least one recombinant imunogenic antigenic determinant, the antigenic determinant being fused to a Hepatitis B virus core antigen and heterologous thereto, and a suitable excipient.

16. The vaccine of claim 16 wherein the immunogenic epitope is from a Plasmodium species.

$(NANP)_4$ 1: 5'- AAC GCT AAC CCG AAT GCT AAC CCG AAC GCT AAC CCG AAC GCT AAC CCG -3'

$(NANP)_4$ 2: 5'- CTA GAC GGG TTA GCG TTC GGG TTA GCG TTC GGG TTA GCA TTC GGG TTA GCG TT 3'

$(DP_4NPN)_2$ 1: 5'- GAC CCG CCG CCG CCG AAC CCG AAC GAC CCG CCG CCG CCG AAC CCG AAC T - 3'

$(DP_4NPN)_2$ 2: 5'- CTA GAG TTC GGG TTC GGC GGC GGC GGG TCG TTC GGG TTC GGC GGC GGC GGG TC -3'

Figure 1

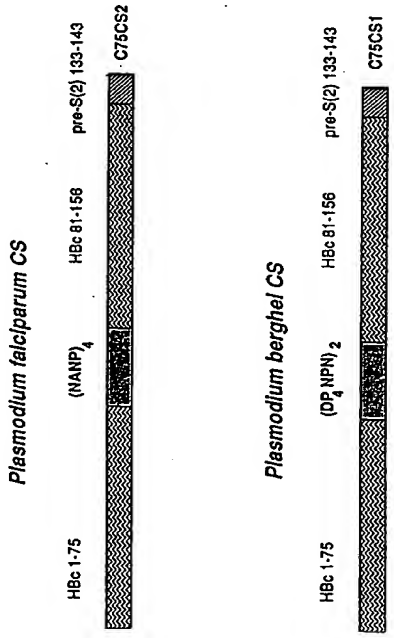


Figure 2

Figure 3

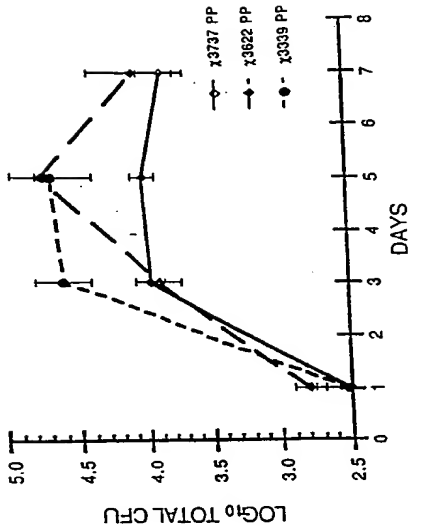


Figure 4

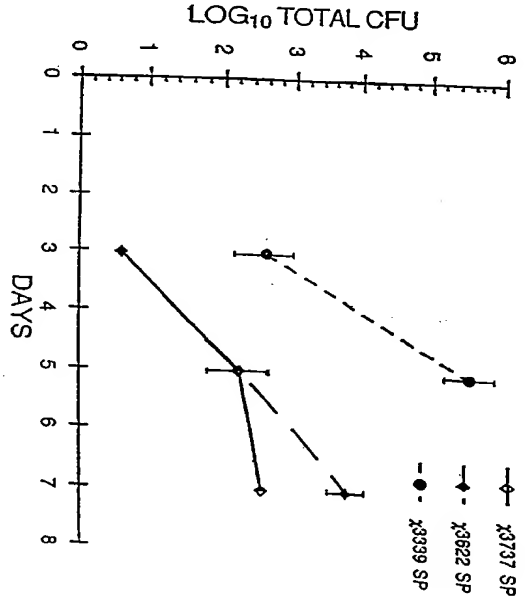
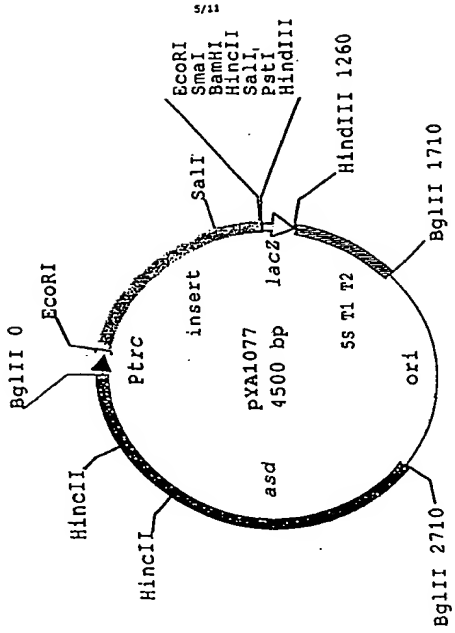


Figure 5



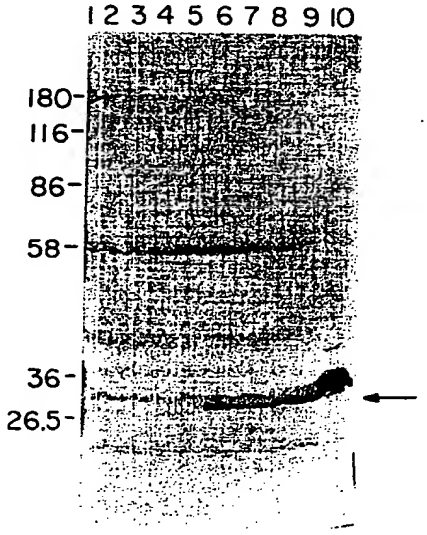


Figure 6

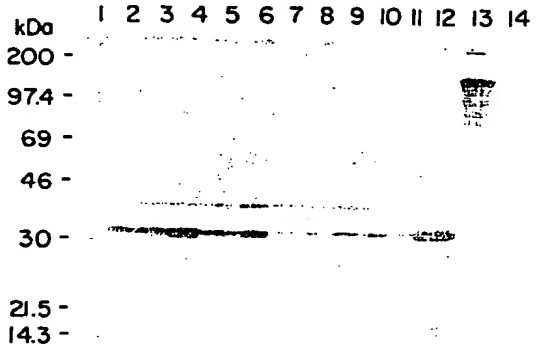


Figure 7

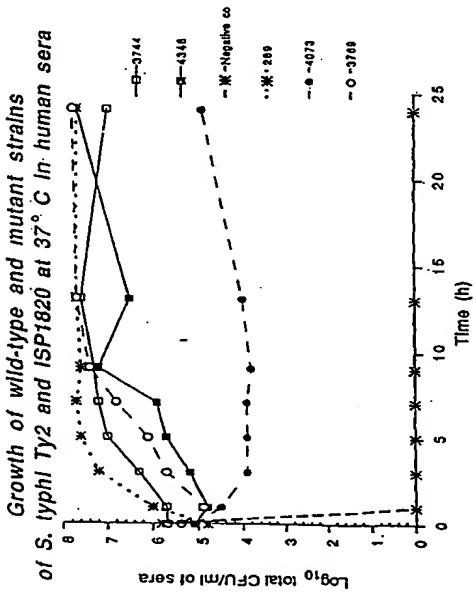


Figure 8

Figure 9

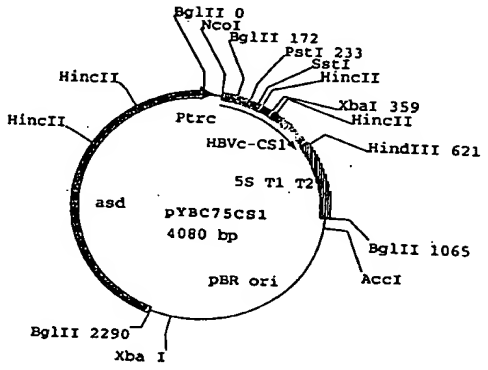


Figure 10

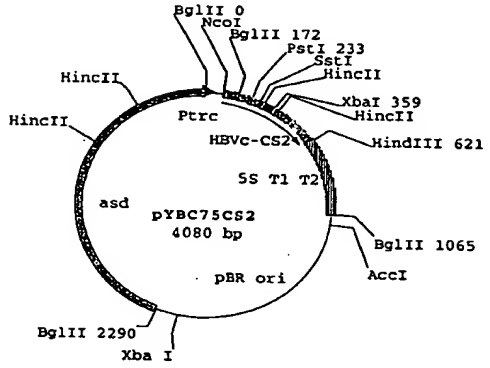


Figure 11

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(57) Abstract

Vaccines and immunogenic compositions which contain at least one immunogenic antigenic determinant, the antigenic determinant being fused to a Hepatitis B virus core antigen and heterologous thereto and methods for making same are provided.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 94/04168

A. CLASSIFICATION OF SUBJECT MATTER
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//(C12N1/21,C12R1:42)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF IMMUNOLOGY., vol.145, no.12, 15 December 1990, BALTIMORE US pages 4317 - 4321 FLORIAN SCHODEL ET AL. 'Hepatitis B virus nucleocapsid/pre-S2 fusion proteins expressed in attenuated Salmonella for oral vaccination' see page 4317, left column, paragraph 1 - right column, paragraph 1 see page 4319, right column, paragraph 2 - page 4320, left column, paragraph 1 --- -/--	1,11,13, 15

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
29 September 1994	18. 10 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Montero Lopez, B

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INTERNATIONAL SEARCH REPORT

International Application No PCT/US 94/04168

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, May 1992, WASHINGTON US page 158 FLORIAN SCHÖDEL ET AL. 'Avirulent Salmonella typhimurium and S. typhi expressing hybrid Hepatitis B virus core/pre-S genes for oral vaccination' see abstract no. E-84	1,11,13, 15
A	WO,A,89 02924 (PRAXIS BIOLOGICS, INC.) 6 April 1989 see page 11, line 33 - page 13, line 30 see page 20, line 21 - page 22, line 6	1-5,8, 11-16
A	WO,A,88 10300 (MEDICO LABS AG) 29 December 1988 see page 2, paragraph 2 - page 4, paragraph 2 see page 4, paragraph 4 - page 6, paragraph 2 see page 14, paragraph 6 see page 15, paragraph 3 -paragraph 4; examples 19,20	1-6, 11-16
A	NATURE, vol.330, no.6146, 26 November 1987, LONDON GB pages 381 - 384 B.E. CLARKE ET AL. 'Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein' cited in the application see page 382, left column, paragraph 2 - page 383, right column, paragraph 1	1,11,13, 15
A	BIOTECHNOLOGY, vol.6, no.6, June 1988, NEW YORK US pages 693 - 697 KOJI NAKAYAMA ET AL. 'Construction of an asd+ expression-cloning vector: stable maintenance and a high level expression of cloned genes in a Salmonella vaccine strain' see abstract see page 694, right column, paragraph 4 - page 695, right column, paragraph 2	1,8-11, 13,15
P,X	JOURNAL OF CELLULAR BIOCHEMISTRY Supplement 18C, 1994, page 232 PATRICIA LONDOÑO ET AL.: 'Use of a double Salmonella mutant to stably express HPV16 E7 protein epitopes carried by the HBV core antigen.' see abstract no. NZ 314	1,11,13, 15

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INTERNATIONAL SEARCH REPORT

(information on patent family members)

International Application No PCT/US 94/04168

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8902924	06-04-89	US-A-	5112749	12-05-92
		AU-A-	2813589	18-04-89
		EP-A-	0383833	29-08-90
		JP-T-	3500246	24-01-91
		OA-A-	9200	30-06-92
WO-A-8810300	29-12-88	AU-A-	1985588	19-01-89
		AU-A-	1995888	19-01-89
		WO-A-	8810301	29-12-88
		EP-A-	0299242	18-01-89
		EP-A-	0304578	01-03-89
		EP-A-	0300213	25-01-89
		JP-T-	2501186	26-04-90
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